A NOVEL ROLE OF THE VACCINIA VIRUS mRNA CAPPING ENZYME IN DETERMINING VIRUS HOST RANGE

by
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Abstract

E3 family proteins are a poxvirus defense gene that have a significant role in host range determination. Their biological function has largely been attributed to the ability of the protein to bind double-stranded RNA. Increasing evidence indicates this mechanism is more complex. For example, the ortholog, SPPV034, is unable to rescue replication of a recombinant virus even though it retains the ability to bind dsRNA.

The Vaccinia virus capping enzyme (the D1-D12 heterodimer) is a cofactor for E3 protein in determining host range. A D1-G801S mutation rescued replication of the SPPV034 recombinant virus and inhibited activation of host responses. A D12-S131N partially rescue replication, but could not fully antagonize host responses. These results indicate that E3 proteins operate on a threshold mechanism determined by intrinsic properties. The D1-D12 heterodimer mutations altered the quantity of dsRNA produced during infections so that the upper limit of the SPPV034 protein was not reached.

Dedication:

For my family.

Who would have thought a girl from a small town in Alberta would have come so far.

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Abbreviations/Glossary:

2'OMTase – 2'O methyltransferase
A549 – adenocarcinomic human alveolar basal epithelial cell line
araC – cytosine arabinoside
BHK21 – baby hamster kidney cell line
CARD – caspase activation and recruitment domains
CMLV – camelpox virus
CPV – cowpox virus
CTD domain – carboxy terminal domain
DAPI – 4',6-diamidino-2-pheylindole
dsDNA – double stranded DNA
DMEM – Dulbecco's modified Eagle's medium
DsRed – Discosoma sp. red fluorescent protein
dsRNA – double-stranded RNA
EGFP – enhanced green fluorescent protein
eIF2α – alpha subunit of eukaryotic initiation factor 2
eIF – eukaryotic initiation factor
EMS – ethyl methanesulfonate
EV – enveloped virion
EVH – ectromelia virus
FBS – fetal bovine serum
GAPDH – glyceraldehyde 3-phosphate dehydrogenase
GTase – guanyltransferase
HBH tag – biotinylation recognition signal flanked by two hexahistidine sequences
HeLa – human cervical cancer cell line
hpi – hours post infection
hpt – hours post transfection
IFN – interferon
IkB – inhibitor of kB
IkK – IkB kinase
IL-6 – interleukin 6

IRF – interferon regulatory factor

ISG – interferon stimulated gene

ITR – inverted terminal repeat

IV – immature virion

L292 cells – mouse fibroblast cell line; derived from C3H/An male mouse

MC – molluscum contagiosum virus

MDA5 – melanoma differentiation-associated protein 5

MEM – minimum essential medium

MOI – multiplicity of infection

MPXV – monkeypox virus

MTase – methyltransferase

MV – mature virion

MVA – modified vaccinia virus Ankara strain

MYX – myxoma virus

NF-kB – nuclear factor kappa-light chain enhancer of activated B cells

NLR – NOD like receptor

OA3.Ts cells – ovine testis cell line

OAS – 2'-5' oligoadenylate synthetase

PACT – PKR activating protein

PAMP – pathogen associated molecular pattern

PARP – poly (ADP-ribose) polymerase

PBS – phosphate buffered saline

PBST – phosphate buffered saline, 0.1% tween

PCR – polymerase chain reaction

Phospho-S6 – phosphorylated ribosomal protein subunit S6

PKR – protein kinase R

PRR – pattern recognition receptor

PVDF – polyvinylidene fluoride membrane

Ribo-S3 – ribosomal protein subunit S3

RIG-I – retinoic acid-inducible gene I

RNaseL – ribonuclease L

RPL11 – ribosomal protein L11

RPL13A – ribosomal protein L13a

RLR – RIG-I like receptor

SDS – sodium dodecyl sulfate

SPPV – sheeppox virus

TLR – toll like receptor

TNFα – tumor necrosis factor alpha

TPase – RNA triphosphatase

UTR – untranslated region

VACV – vaccinia virus

VARV – variola virus

VITF – vaccinia virus intermediate transcription factor

VTF – vaccinia virus termination factor

WHO – World Health Organization

WV – wrapped virion

Chapter One: Introduction

1 Introduction to Poxviruses

1.1 The Poxviridae

The family *Poxviridae* comprises large enveloped viruses with dsDNA genomes and cytoplasmic sites of replication[1]. Historically, poxviruses have been significant in a number of scientific break-throughs. *Variola* (VARV) was the cause of smallpox, a disease that drastically influenced human history. It is estimated that smallpox was responsible for over 500 million deaths during the 20th century[2]. Edward Jenner revolutionized public health when he demonstrated that cowpox virus could be used to protect individuals from smallpox[3]. The World Health Organization (WHO) initiated a global vaccination program in 1959 using *Vaccinia virus* (VACV) that lead to the eradication of smallpox in 1979[4]. Smallpox remains the only human pathogen ever eradicated. Other major scientific discoveries involving VACV include being the first animal virus to be viewed using a light microscope, first virus grown in culture, contributions to infectious disease/immunity concepts and elucidation of the structural features of viral and eukaryotic mRNA (including the 5'-cap and 3' poly(A) tail)[1]. Continued work with poxviruses as live recombinant expression vectors provide a powerful tool for the study of immunological mechanisms and alternative vaccines for a myriad of other pathogens and cancer.

Poxviruses contain a single, linear dsDNA molecule with inverted terminal repeats (ITRs). The genome and ITRs vary in size across species. However, researchers have identified nearly 100 genes that are conserved in all chordopoxviruses (poxviruses capable of infecting vertebrates)[5]. The arrangement of genes in chordopoxviruses is also remarkably similar, with those highly conserved genes (those for replication functions) are usually centrally located, while variable genes (those for host interactions) are located in the end regions. The genome of VACV is shown in Figure 1.1.

The receptor for poxvirus entry is unknown, but viruses have been shown to be capable of entering a large variety of cell types. Therefore, modes of transmission for orthopoxviruses may include respiratory (as is the case for VARV, VACV and monkeypox virus)[6–8] or direct contact with infected individuals/animals or contaminated fomites[9].

1.2 Entry, gene expression, and genome replication

As mentioned above, the receptor for cellular entry of poxviruses remains to be discovered. Poxvirus virions exist in two forms, a mature virion and an enveloped virion, which make the mechanisms for entry difficult to investigate. Further hindering our understanding of the fusion mechanisms is that the structure of the entry-fusion complex remains unresolved. However, strides have recently been made in identifying viral membrane proteins for attachment/entry[10] and interacting partners from the host[10,11]. Eight viral membrane proteins (A16, A21, A28, G3, H2, J5 and L5) were identified as necessary for the formation of a stable entry/fusion complex. Interestingly, these polypeptides traffic independently to the viral membrane indicating a complex interaction network. Additionally, it was revealed that strong fusion between virus and cells in culture occurs at acidic pH, indicating endocytosis has a prominent role in viral entry.

Previous studies have suggested that the mature virion membrane contains all the proteins necessary for the fusion complex[1]. Therefore, any enveloped virions that attach to a cell will shed this outer membrane[12], exposing the mature virion membrane for the formation of the entry complex. Entry mechanisms also account for the diversity of cells capable of being infected, and can occur directly through plasma membrane fusion, or following endocytosis[13–16]. Following endosome acidification, the virus core is released into the cytoplasm, where gene expression and genome replication can proceed[17–19]. Actin rearrangements have also been suggested for the initial fusion of membranes[18,20].

An interesting feature of poxviruses is the temporal regulation of genes. Gene expression occurs in three phases (Figure 1.2). Early gene expression occurs within the first hour of infection, prior to genome replication, and consists of enzymes for viral DNA synthesis, transcription factors for intermediate genes and host immune system modulation/evasion factors[1]. Intermediate and late gene expression follows genome replication and occurs between 1 and 3 hours post infection, using progeny genomic DNA as template. Intermediate proteins are associated with DNA binding/packaging and late transcription factors[21], while late genes encode virion structural proteins (including the entry-fusion complex) and early gene transcription factors that get packaged into the virion core[22]. Some researchers have suggested that post replicative gene transcription may occur due to inaccessibility to the genome until after

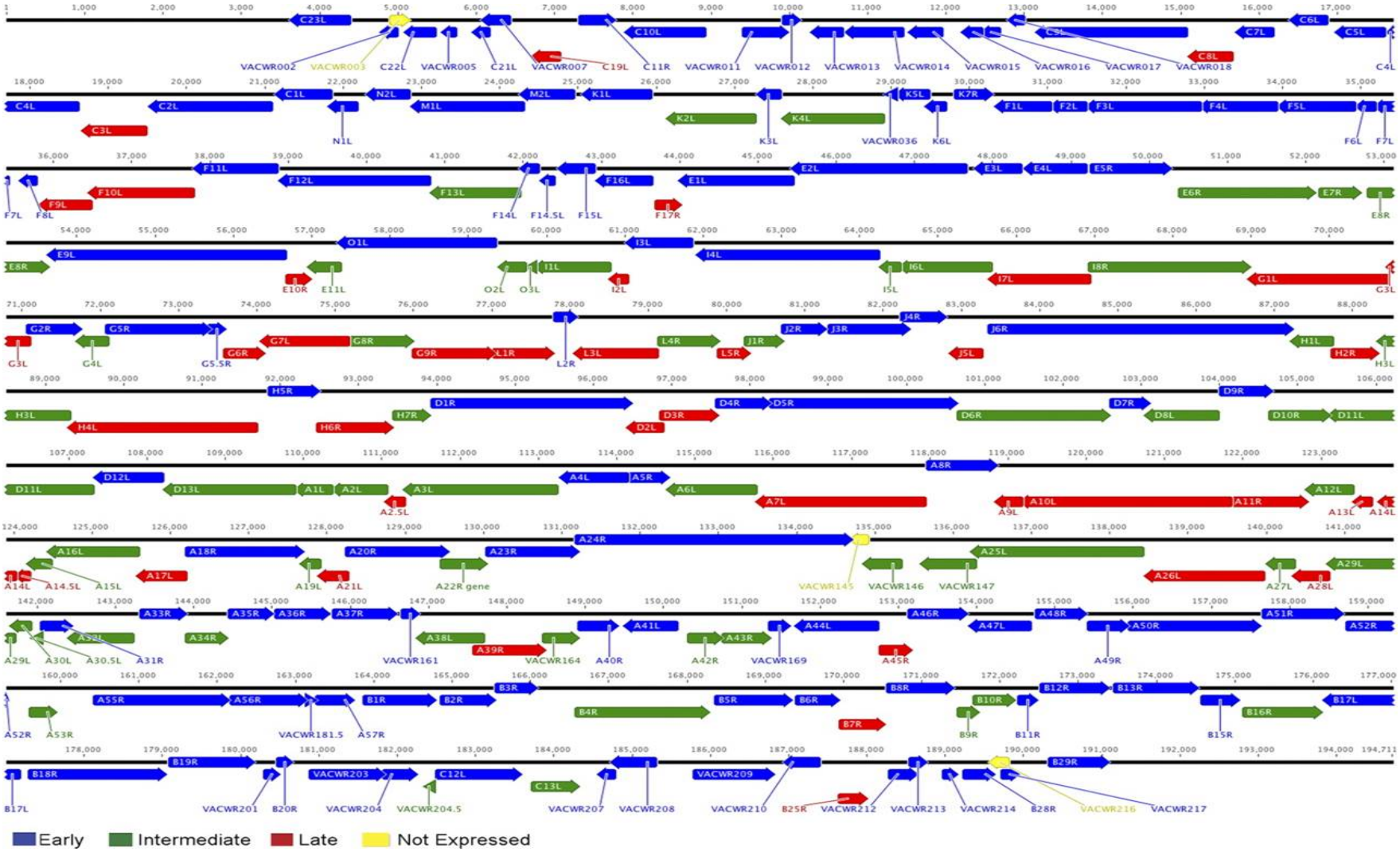

Figure 1.1 VACV genome map.
Early genes are highlighted in blue, intermediate genes are highlighted in green and late genes are highlighted in red. Non-expressed genes are highlighted in yellow. Reproduced with permission from American Society for Microbiology. (From Yang Z, Reynolds SE, Martens CA, et al. Expression profiling of the intermediate and late stages of poxvirus replication. J Virol 2011; 85:9899-9908.)

DNA replication occurs[21]. Poxviruses encode their own RNA polymerase and transcription factors, however, certain cellular proteins are also required for intermediate gene expression[23].

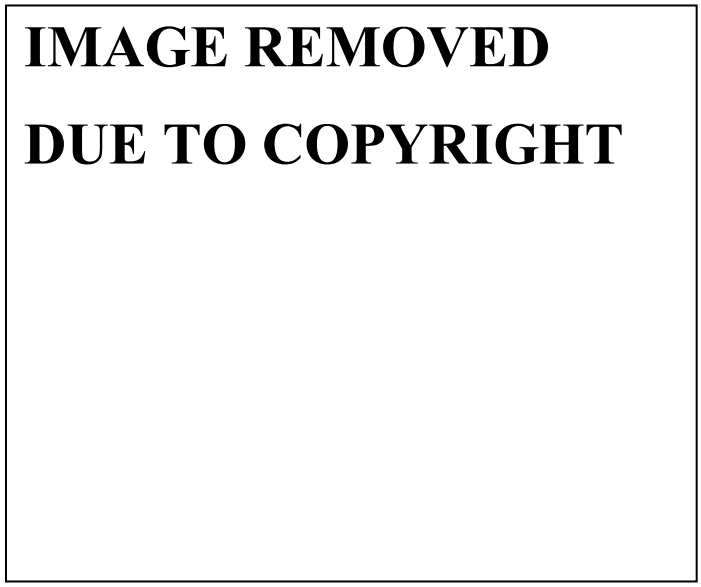

Figure 1.2 Poxvirus gene expression occurs in three distinct stages.
Early transcript expression occurs between 0-2 hours post infection, intermediate transcript expression occurs between 1-3 hpi and late transcript expression occurs between 2-4 hpi. Graph is based on steady state levels of mRNA encoded by C11R (early), G8R (intermediate) and F17R (late) ORFs. Reproduced with permission from Wolters Kluwer Health. (From Moss B. Poxviridae: the viruses and their replication. *Field's Virology* 7th Ed .)

Genome replication begins between 1-2 hours post infection in the cytoplasm of infected cells. Discrete foci of replication (also known as virus factories) have been observed[24,25] and results in 10,000 genome copies per cell, with approximately half of these being packaged into infectious progeny[26,27]. Poxvirus researchers have proposed two mechanisms for genome replication. One is similar to the rolling displacement mechanism, where a nick occurs and the replicating DNA strand folds back on itself granting access to the replication complex[28,29]. However, viral DNA nicking enzymes and nicking sites within the viral genome have not been identified to date. The other mechanism proposes semi-discontinuous synthesis using replication forks and RNA priming (Figure 1.3). The latter method has become widely accepted after the recent identification of a viral RNA primase and the discovery that poxviruses hijack a cellular ligase[30–33]. The full life cycle of poxviruses is summarized in Figure 1.4.

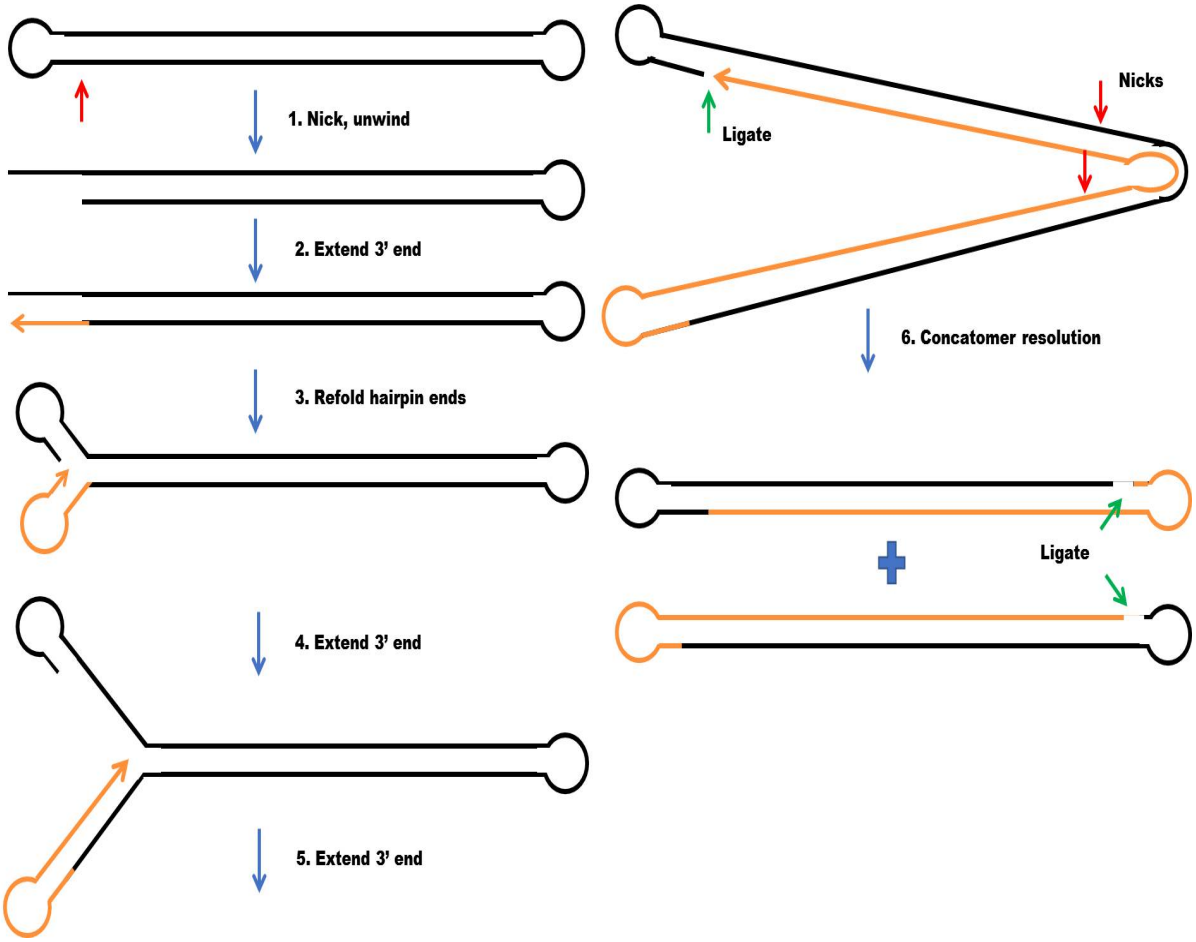

Figure 1.3. Self-priming model of POXV DNA replication.
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Another interesting feature of poxviruses is the active occurrence of recombination events during genome replication. It has been suggested that the viral DNA polymerase plays an important role in the recombination mechanism[34–36]. Researchers regularly take advantage of these events to create recombinant viruses using transfected sub-genomic DNA fragments[37]. In this regard, poxviruses can be used as expression vectors for genes of interest and for the generation of recombinant vaccines.

1.3 Generation of dsRNA

Double-stranded RNA (dsRNA) is frequently produced during virus infections while remaining largely absent from the cytoplasm of uninfected eukaryotic cells. Therefore, dsRNA is

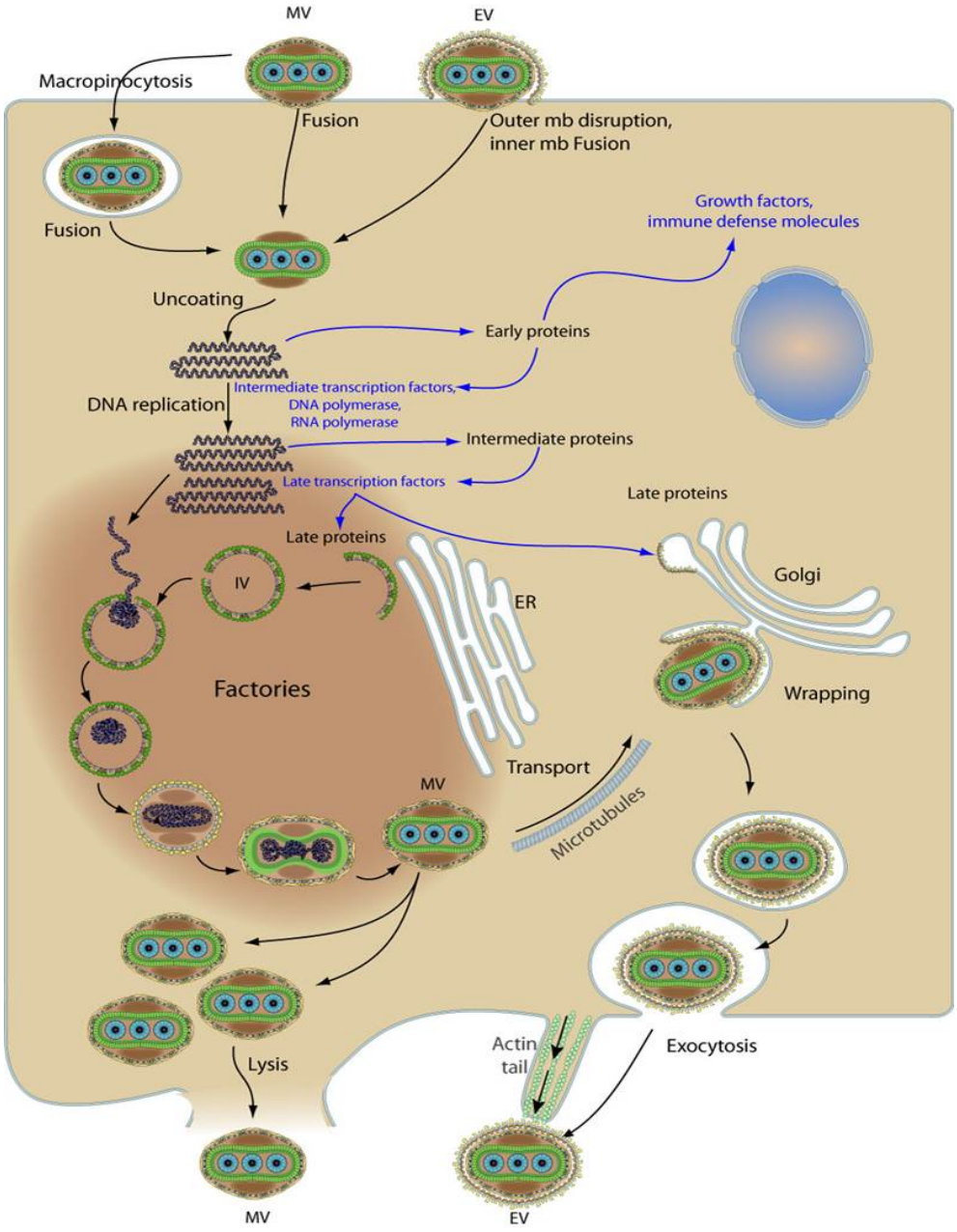

Figure 1.4 POXV life cycle.
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a potent activator of innate immunity[38], and mammalian cells have several mechanisms for dsRNA detection including the protein kinase R (PKR) and 2'-5'-oligoadenylate synthetase (OAS) systems[39,40]. This has created an evolutionary arms race, with viruses developing multiple methods of masking dsRNA produced during infection for evasion, which will be discussed later.

For poxviruses, dsRNA is generated mainly from convergent intermediate/late transcription processes. Poxviruses possess two mechanisms for the termination of RNA transcripts. Early transcripts contain termination sequences at the 3' ends and efficient cleavage occurs downstream of these signals[1]. These sequences are not recognized during post-replicative transcription and undergo site-specific cleavage instead[41,42]. Consequently, intermediate and late transcripts contain long, heterogeneous 3' ends which have the potential to form dsRNA structures, as poxvirus genes exist in both orientations. A schematic for the generation of dsRNA products during VACV replication is shown in Figure 1.5.

1.4 Structure and morphogenesis

The uniqueness of poxviruses extends to their virion structure and morphogenesis. Poxvirus morphogenesis is summarized in Figure 1.4. Infectious particles exist in two forms. The mature virion (MV) is the more abundant form and consists of a single brick-shaped membrane surrounding a biconcave virus core containing the virus dsDNA genome and early enzymes[43]. These particles are released via cell lysis only[43]. The enveloped virion (EV) is the MV wrapped in an additional membrane for efficient cell-to-cell spread. The majority of EVs remain attached to the cell surface, utilizing actin rearrangements to form tail-like structures for the propulsion of particles to neighbouring cells[44,45]. Released EVs are essential for long-range dissemination of the virus[46]. Preventing fusion of newly assembled/released virions also allows for increased dissemination of the virus within an infected host[47].

Poxvirus virion assembly occurs via a process that is exclusive to this family of viruses. The distinct structures that occur during morphogenesis are summarized in Figure 1.6. Initially, membrane proteins conjugate in a crescent shape, which will eventually fold together to form a spherical, immature virion (IV). Virus genetic material and early enzymes enter the IV before it seals[48–50]. The IV then transitions into the distinct barrel-shaped MV through the disassembly of a specific scaffold protein (D13)[51–53]. Some MVs are transported to the Golgi apparatus of host cells where they are wrapped in a double membrane[54–56]. These structures are referred to as wrapped virions (WV). WVs are transported to the plasma membrane where the EV is released by exocytosis via the fusion of the outermost membrane.

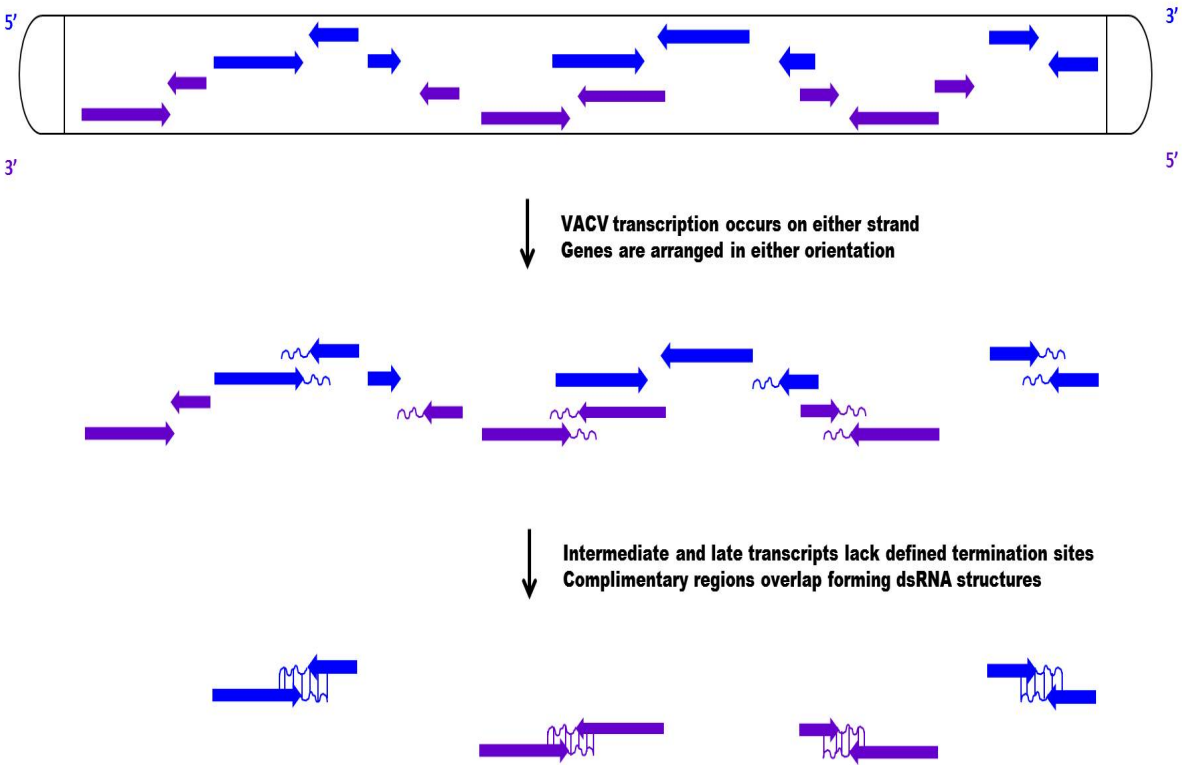


Figure 1.5. Generation of dsRNA products during VACV transcription.
Poxvirus genes occur on either strand of genomic DNA and are oriented in both directions. During intermediate and late gene transcription the RNA polymerase generates heterologous 3' ends as these genes lack defined termination sequences. Complementary sequences will overlap and generate dsRNA structures.

1.5 Vaccinia Virus as a model organism

VACV is the most comprehensively studied of the orthopoxviruses based on its attenuated pathogenicity and similarity to VARV. These features were critical for the use of VACV as a live vaccine that led to the eradication of smallpox in 1980. Since VARV and monkeypox virus research require higher containment facilities, VACV is routinely used as the model instead to study the basic biology of the virus. The fact that the origins of VACV and its reservoir remain unknown is curious considering its effectiveness as a vaccine and the immense research since its discovery[57]. Despite its attenuated pathogenicity compared to VARV, VACV vaccinations have the highest rate of adverse reactions[58] among all the human vaccines. Since the dismantling of the VACV vaccination program, there has been a surge in the number of poxvirus outbreaks owing to the naivety of the population[59]. Therefore, uncovering the mechanisms used by poxvirus for pathogenicity is critical for the development of therapeutic agents.

VACV also possesses immense genome plasticity and is easily manipulated using recombination mechanisms (see above). This has led to the development of modified strains for use as recombinant expression vectors. Recombinants are frequently generated for protein synthesis, vaccine candidates and potential cancer therapies[60,61]. The modified vaccinia Ankara (MVA) strain is the most widely studied/tested vaccine vector candidate[62,63], however, safety concerns restrict its use to clinical trials. Understanding poxvirus-host interactions will be fundamental to alleviating concerns, optimizing efficacy and limiting side effects of future vaccine vector candidates.

2 Virus Host Range Determination and Immune Evasion Strategies

2.1 Virus host range

Host range of poxviruses also varies with species. VACV has no known natural host and can infect a number of different mammal species including humans, rodents, rudiments and non-human primates[1]. VARV, on the other hand, was the only orthopoxvirus that was not zoonotic and a human-only pathogen. Since poxviruses are able to enter a number of different cells, the inhibition of the subsequent viral replication process is usually what determines viral host range[64]. A number of host-virus interactions have been implicated in the virus-host range determination. Most frequently, it is viral defense genes and their mechanisms, which are specific to a particular genus or species, that have the most significant influence on tropism and even pathogenicity.

2.2 Innate immune responses to viruses

Innate immunity is based on the fundamental principle of using host cell receptors (known as pattern recognition receptors, PRRs) to recognize molecular motifs conserved among all pathogens (known as pathogen-associated molecular patterns, PAMPs)[38]. This system is an organism's first and most critical response to infection as it will be the driving force that dictates direction and amplitude of adaptive immunity[65,66].

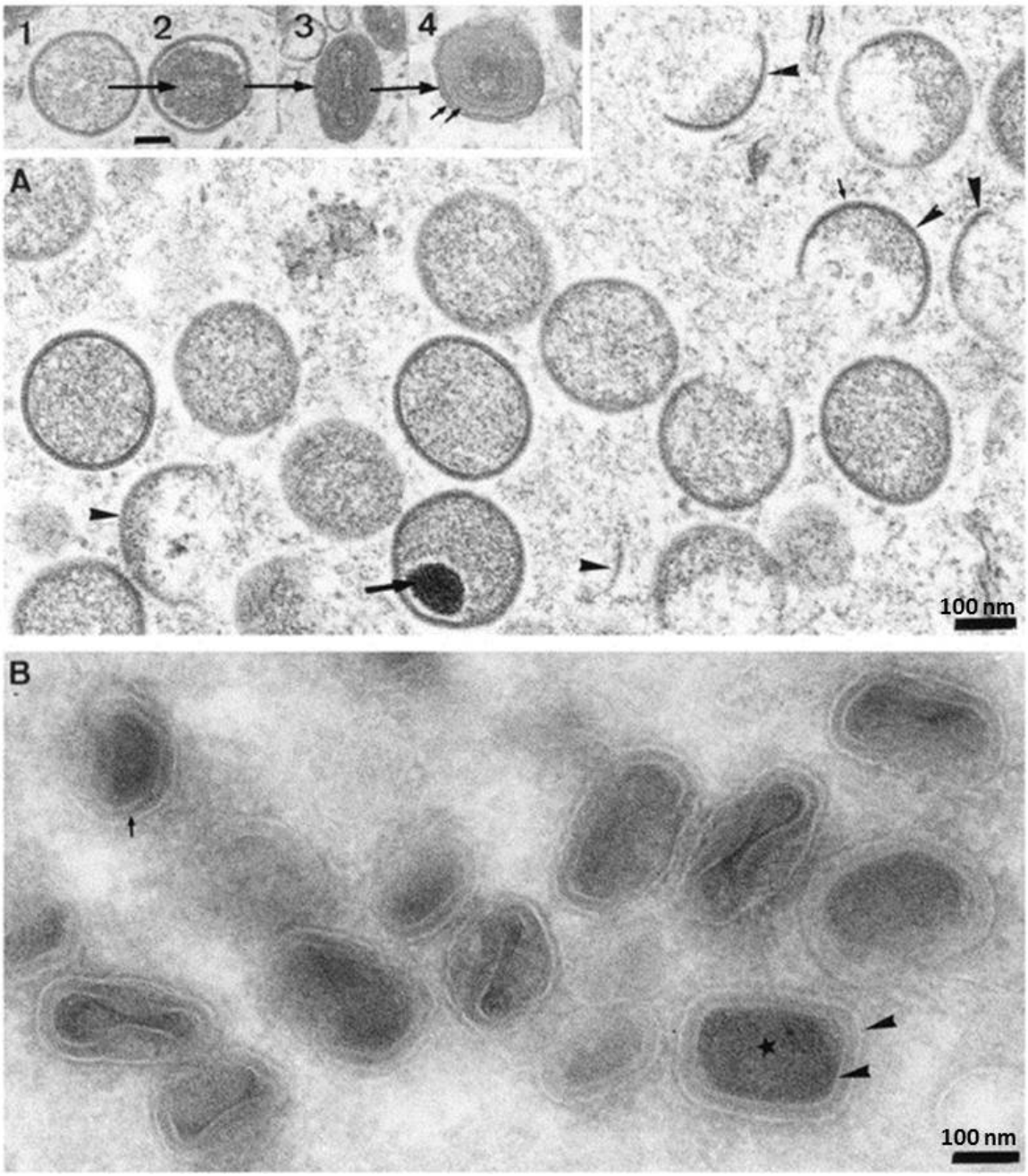


Figure 1.6 EM imaging of VACV morphogenesis.
A, inset) Consecutive stages of VACV assembly: (1) spherical immature virion, (2) spherical intermediate, (3) mature virion, (4) enveloped mature virion. A) Section displaying the development of crescent structures (arrow heads) and spherical immature virions. Dense nucleiod of DNA with an IV is also depicted (large arrow). B) Section displaying the development of mature virions. The classic brick-shaped profile (star) and the double membrane of the wrapped mature virion (arrow heads) are depicted. Reproduced with permission from American Society for Microbiology. (From Griffiths G, et al. Structure and assembly of intracellular mature Vaccinia virus: thin-section analyses. J Virol 2001; 75:11056-11070.)

Innate pattern recognition receptors have been classified into three groups. NOD-like receptors are important for recognizing lipopolysaccharides and other structures of the cell wall of bacteria (NLRs), while Toll-like receptors (TLRs) and Rig-I-like receptors (RLRs) recognize nucleic-acid based PAMPs such as single-stranded or double-stranded RNA species (ssRNA or dsRNA, respectively). Cellular versions of these structures do not activate PRRs as they are either sequestered in cellular compartments or contain structural modifications that prevent interactions. Therefore, any interaction between PRRs and a PAMP is indicative of a foreign invader, resulting in signalling cascades for the activation of innate defences. Consequently, the coevolution of hosts and pathogens has allowed viruses to develop elaborate mechanisms to evade detection.

i. Nucleic acid detection by RIG-I like receptor signaling

RLRs are intracellular receptors and have been categorized into three types; RIG-I, MDA5 and LPG2. Unique dsRNA structures are ligands for RIG-I and MDA5 and upon recognition, activate signalling cascades to induce antiviral responses, while LPG2 has been shown to be a regulator of RLR signalling[67,68]. RIG-1 and MDA5 share a similar function and structure with two tandem CARD repeats at the N-terminus followed by a DExD/H box RNA helicase and an ATPase domain[69]. These two receptors differ at the C-terminus, with RIG-I containing a repressor domain. On the other hand, LPG2 also contains a DExD/H RNA helicase and ATPase domain but lacks both the CARD and repressor domains[70].

Ligand specificity of RIG-I and MDA5 has been well characterized. RIG-I is preferentially activated through binding of short dsRNA molecules (less than 100bps) bearing a 5'-triphosphate[71,72], but can also recognize longer dsRNA species[73]. Studies have shown that adding an m7-cap to short dsRNA molecules abolishes RIG-I activation[74]. Whereas MDA5 recognizes long dsRNA molecules (3-4kbps) and the presence of a 5'-triphosphate is not necessary for MDA5 activation[75]. Since eukaryotic mRNA is synthesized with a 5'-m7-GTP cap, this provides an important means for discriminating self and non-self RNA molecules. Although the intricacies of the virus-host interaction have evolved methods for the masking of viral 5'-triphosphate, which will be discussed later.

The mechanism for RIG-I receptor signalling has been studied extensively, although much less is known about MDA5 or LPG2. The binding of dsRNA to the C-terminus of RIG-I liberates the interaction between the repressor and CARD domains[76]. Activation of RIG-I ultimately leads to the formation of a signalling complex for the expression of transcription

factors NFkB, IRF3 and IRF7 for the secretion of various cytokines and chemokines, including IFN-β, to induce an antiviral state[77–79]. *In vivo* work with LGP2 indicates it acts as a positive regulator and facilitates RLR signalling by modifying RNA structure and localization during infections[80,81].

The importance of RLR signalling can be highlighted through the demonstration that many viruses encode proteins to inhibit its activation. This includes proteins that target regulators both upstream and downstream of the RLR signalling complex[82–84]. Many viruses also encode dsRNA binding proteins to interfere with these molecules and prevent activation of the RLR pathway altogether[85]. The signaling pathway of RLRs is outlined in Figure 1.7.

ii. Induction of antiviral response by protein kinase R

The presence of dsRNA in the cytoplasm of eukaryotic cells is a major indicator of infection. In this regard, the innate immune system has numerous enzymes for dsRNA detection. Another PRR dedicated to dsRNA surveillance is dsRNA activated kinase, also known as protein kinase R (PKR). PKR is comprised of 2 tandem dsRNA binding motifs at the N-terminus, followed by a C-terminal serine/threonine kinase domain[86] which forms a homodimer when activated[87]. Basal expression of PKR in most cells is sufficient for the detection of virus infection, but augmented expression is induced by IFN signalling from neighbouring cells. Activation of PKR occurs through binding of dsRNA with efficient activation occurring when dsRNA molecules are between 55bps and 2kb[88,89]. Length of dsRNA is crucial for PKR signalling as a single molecule must be able to bridge the dsRNA binding motifs of both monomers for activation to occur[86]. Like RIG-I, PKR can also be activated by shorter dsRNA structures (formed by ssRNA secondary structures). However, this activation is dependent on the length of the ssRNA tail and the presence of the RNA 5'-triphosphate[90]. Additionally, PKR may be activated by protein-protein interactions between dsRNA binding domains by PKR-activating protein (PACT)[91].

PKR acts on numerous downstream effectors that influence numerous cellular processes. The eukaryotic initiation factor 2 alpha (eIF2α) substrate of PKR has been studied extensively. Following dsRNA binding, the kinase domain of PKR undergoes autophosphorylation and subsequently restricts protein translation through phosphorylation of the ribosomal subunit eIF2α (Figure 1.8). The initial reaction in protein synthesis is the hydrolysis of eIF2α bound GTP to GDP[92]. Phosphorylated eIF2α is unable to recycle GDP to GTP, thereby keeping eIF2α in an inactive state and preventing successive rounds of translation from initiating[93].

Control of virus replication has been linked to PKR activation in VACV[94], Hepatitis C virus[95,96] and West Nile virus infections[97]. Therefore it is not surprising that many viruses also employ RNA and protein inhibitors to modulate PKR activation. These inhibitors are primarily categorized into three groups based on their mechanism of action. The first group are protein inhibitors of PKR (such as Ebola VP35[98] and influenza NS1[99]). These are suggested to function by direct interactions with PKR or indirectly by employing dsRNA binding proteins to sequester viral dsRNA products. However, many dsRNA binding proteins are also known to mediate protein-protein interactions. Therefore it remains to be determined precisely whether inhibition of PKR is a result of dsRNA binding, protein-protein interactions or a combination of methods. The second group of PKR inhibitor are small dsRNA molecules. This method of inhibition is used by adenovirus and Epstein-Barr virus. These small dsRNA molecules are deliberately expressed[100] to 'clog up' PKR proteins and prevent bridging of the two monomers. The third group of PKR inhibitors are eIF2α pseudo-substrate. For example, VACV encodes the K3 protein, which is structurally similar to eIF2α; however, its role remains unclear as its function appears to be overshadowed by the expression of E3 protein, at least in cell culture systems[101,102].

iii. Response to viral infections by interferons

An important feature of innate immunity is the secretion of small proteins, called cytokines, which are vital for the communication between neighbouring cells and between immune system effector cells. Cytokines include a diverse array of proteins including tumour necrosis factors (TNF), interleukins (IL) and other growth factors. One of the most important families in the generation of an antiviral response is the interferons (IFN), which are categorized into type I, II, or III[103]. As described above, RLR signalling results in secretion of Type I IFNs (IFNβ and IFNα), but has also been shown to activate type III[104].

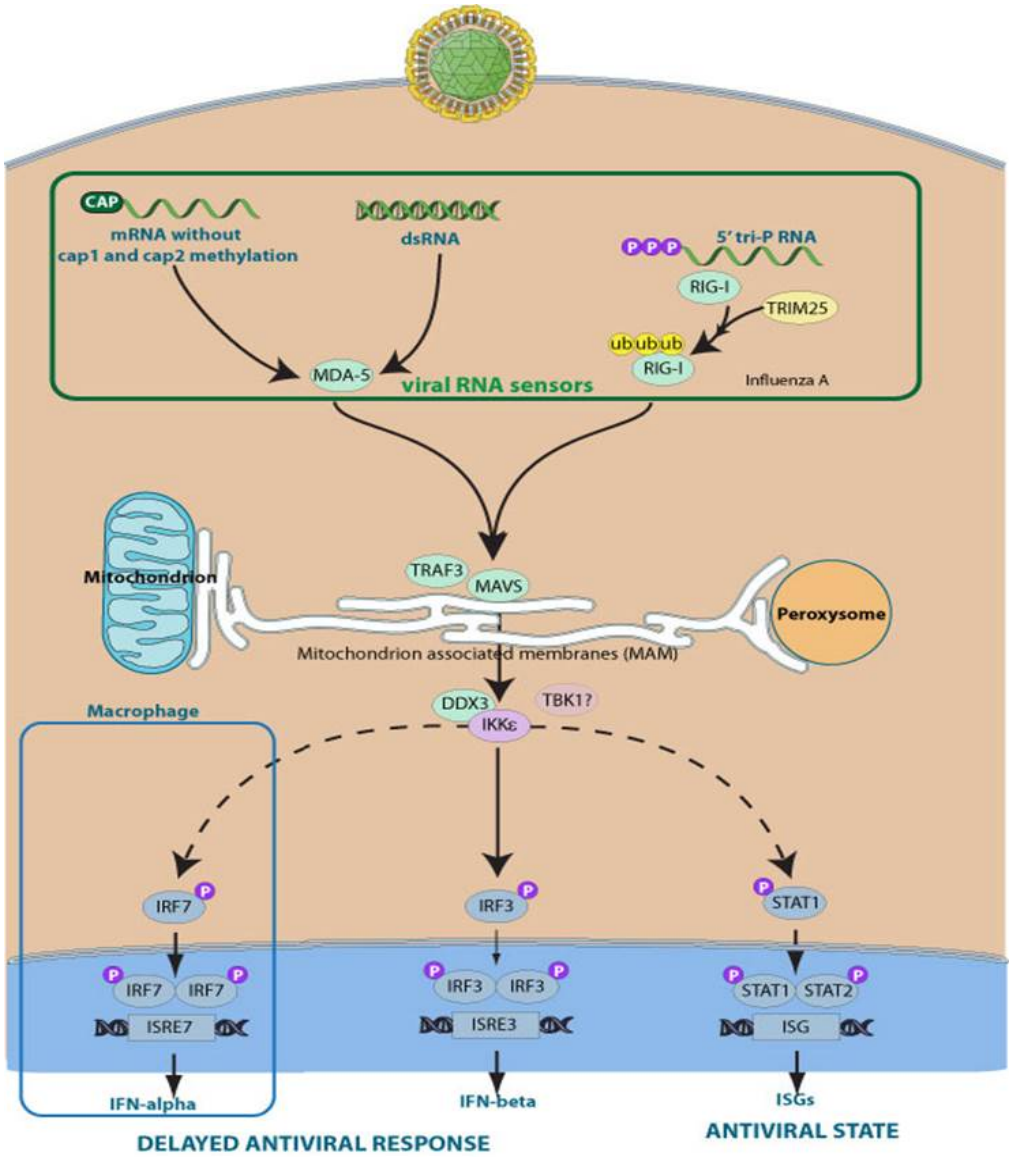


Figure 1.7 RIG-I like receptor signaling and antiviral responses to detection of viral RNA species. Adapted from https://viralzone.expasy.org/553. Used with permission from SIB Swiss Institute of Bioinformatics.

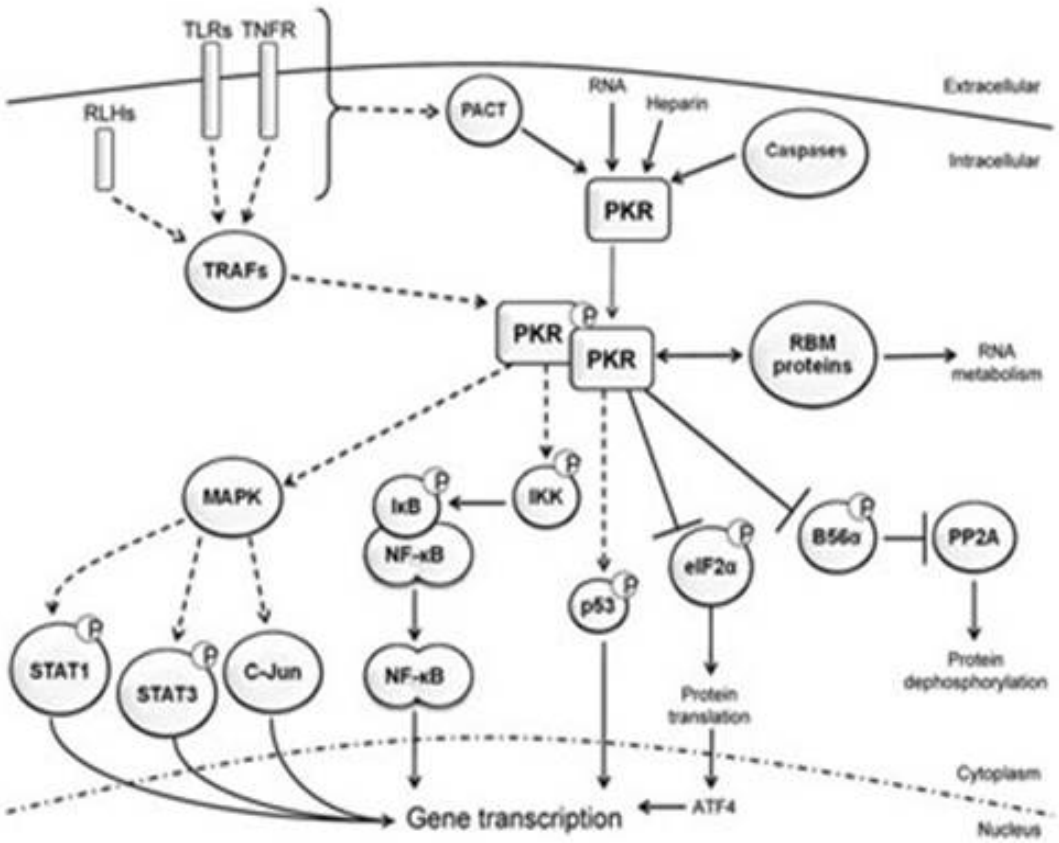


Figure 1.8. Protein kinase R signaling.
PKR is activated through binding of dsRNA structures which induces dimerization and auto-phosphorylation. PKR functions directly by protein phosphorylation (solid lines) or indirectly by integrating signaling networks to promote the activation of transcription factors for antiviral response gene transcription (dashed lines). This figure is representative of PKR function and does not include all protein substrates and pathways. Reprinted with permission of Mary Ann Liebert, Inc. Publishers. (From: Pindel and Sadler. The role of protein kinase R in the interferon response. *J Interferon & Cytokine Res* 2011; 31:59-70)

Cytokines, especially IFNs play pivotal roles in establishing an antiviral response. The mechanism for IFNβ signalling has been considerably investigated. Specifically, IFNβ binds the heterodimeric type I IFN receptor on neighbouring cells[105,106] which initiates a signalling cascade culminating in the formation of the IRF9/ISGF3 complex[107]. This complex enters the nucleus where it acts as a transcription factor for hundreds of IFN stimulated genes (ISG). The cell then enters an antiviral state, viral replication is blocked and destruction of infected cells is promoted. The signaling pathway for IFNs is summarized in Figure 1.9.

One ISG studied at length is PKR, however, the mechanism in which PKR contributes to cytokine expression remains unclear. The expression of several transcription factors has been linked to PKR via its interaction with the IkK complex of the NFkB pathway[108,109]. PKR-mediated activation of IkK results in phosphorylation of the NFkB repressor, IkB, marking IkB

for degradation, thereby freeing NFkB to enter the nucleus. Furthermore, Schulz *et al.* reported that PKR is able to sustain IFNβ production by stabilizing mRNA transcripts[110].

iv. Responses to viruses using programmed cell death

Cytokines can also be involved in apoptosis, a mechanism of programmed cell death in which infected cells are destroyed. Caspases are a family of apoptotic proteins that undergo sequential activation through cleavage of upstream caspases. Apoptotic signalling occurs via two pathways. The intrinsic pathway involves the permeabilization of the mitochondrial membrane and release of cytochrome C to impair cellular functions, cleavage of caspases 3 and 7, and eventual cell death[111]. The extrinsic pathway is activated when the cytokine TNFα binds to TNF death receptors. Dimerization of death receptors trigger cleavage of caspase-8[112]. The subsequent signalling cascade results in the cleavage of effector caspases (caspase-3 and caspase-7) trigger genomic DNA fragmentation, condensation of chromatin and cell death.

Interestingly, several pro-apoptotic pathways have been linked to activation of PKR[113]. Specifically, PKR-dependent apoptosis has been linked to dsRNA activation of TNF-receptor induced apoptosis via an unknown mechanism involving eIF2α[114,115]. Additionally, PKR-dependent apoptosis is also linked to PKR-induced expression of NFkB and its regulatory role of apoptosis.

2.3 Vaccinia virus strategies to inhibit innate immune responses

The interaction between VACV and the innate immune system is an intricate balancing act. Viruses rely on various cellular machinery for replication. However, the defence systems the host relies on for protection must be avoided/inhibited to establish a permissive environment for viral growth. Poxviruses are known to encode numerous proteins that function to suppress innate and adaptive immunity[116]. The nature of this relationship is complicated further by the redundancy of a large number of these immune modulators.

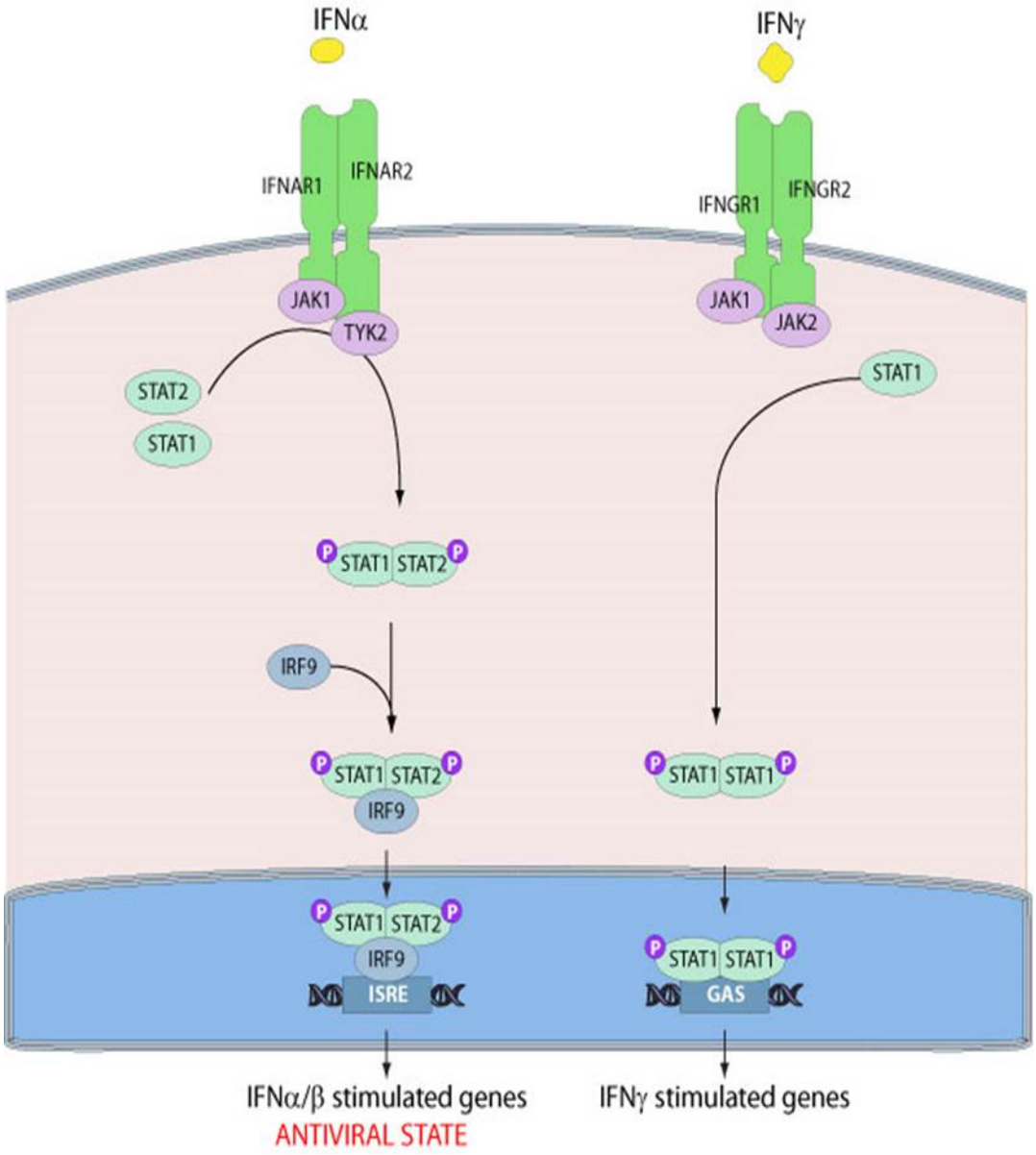


Figure 1.9 IFN receptor signaling.
Adapted from https://viralzone.expasy.org/843. Used with permission from SIB Swiss Institute of Bioinformatics.

The importance of NFkB was presented earlier by outlining the abundance of signalling pathways culminating in its activation. The significance of NF-kB is also outlined by the large number of VACV proteins that target this pathway. For example, VACV employs K1 and B14 to prevent degradation of the NFkB repressor, IkB, through different mechanisms[117,118]. Since cytokines are essential both in the generation of innate immune responses and focusing adaptive immune responses, VACV encodes several receptor homologues to prevent downstream

signalling[116]. Also, several immune modulator proteins have multiple functions and targets in cellular immunity. One such protein that has been rigorously investigated is the E3 protein.

i. E3 protein

The E3L gene encodes an early stage protein that consists of an N-terminal Z-DNA binding domain and a C-terminal dsRNA binding domain[119]. The functions of this protein include regulating antiviral defence, particularly through inhibition of PKR[120], and facilitating viral replication. Studies involving each domain have revealed that the N-terminus is dispensable for virus replication *in vitro*, however, it is essential for pathogenicity *in vivo*[121]. This is an interesting finding since the majority of the E3 immune modulation functions *in vitro* have been mapped to the C-terminus.

Experiments using an E3L-knockout virus display narrowed cellular tropism, failing to replicate in HeLa, Vero, or L929 cells[122,123]. VACV resistance to IFNs is also a result of E3 protein function[124], and E3 protein has been used to confer IFN resistance to other viruses[125]. This precise mechanism has yet to be elucidated, however, it has been suggested that E3 protein acts to block various ISGs[126,127] and IFN effects on replication of sensitive E3L-knockout virus are mediated through PKR activation[128]. Additionally, several other dsRNA binding proteins have been shown to complement E3 function[129–131]. However, recent work with poxvirus E3 orthologs[132] and E3 mutagenesis[133] suggests that the biochemical activity of dsRNA binding is not the basis of the biological function of the E3 protein.

ii. PKR antagonism

Initial studies into E3 protein function determined it has potent activity in inhibiting PKR activation[120]. Historically, PKR inhibition has been attributed as the primary function of E3[119,120]. Since E3L-knockout viruses abort replication during intermediate protein synthesis, a model in which PKR suppresses replication via activation of eIF2α was widely accepted. However recent work with E3 ortholog chimeras has demonstrated VACV replication even when PKR is activated (*Varga and Cao; manuscript in preparation*). Additionally, mutagenesis of E3 residues critical for dsRNA binding revealed a mutation that eliminated the ability of E3 to bind dsRNA, but this mutant retained the ability to replicate in HeLa cells, albeit at a slower rate[133]. Therefore, the exact mechanism for E3 protein's function remains to be clarified.

Likewise, the mechanism for PKR inhibition by E3 protein is also not well defined. The widely accepted model proposes that E3 protein sequesters the dsRNA ligands generated from cytoplasmic virus replication. However, there is mounting evidence that contradicts this theory.

Both the dsRNA binding domain and the kinase domain of PKR was shown to interact with E3[134,135]. This area of the PKR kinase domain has also been suggested as the interface between PKR and eIF2α. Furthermore, the dsRNA binding domain of E3 is also required for regulation of protein-protein interactions[134]. Therefore, it has been difficult to separate these two functions of E3 experimentally. It is also possible that E3 functions in some unrelated mechanism yet to be discovered.

iii. Cytokine modulation

Pathways for the expression of antiviral cytokines are targeted by E3 protein in a PKR-independent manner. Initial investigations of IFNβ expression determined that E3 protein blocks IRF3 and IRF7 induction, thereby abolishing downstream signalling of the RLR pathway[136,137]. Further experiments with E3L-knockout virus infections revealed that VACV induces TNFα and IL-6 expression[138]. Therefore E3 protein must also have a role in their suppression. Inhibition of TNFα was linked to the dsRNA binding abilities of E3 protein[139], and experiments using E3 orthologs have demonstrated that suppression of TNFα and IL-6 is also independent of PKR activation[132].

Although experiments with an E3L-knockout virus have correlated cytokine expression with activation of PKR, p38, IRF3 and NFkB pathways, direct evidence from infections in murine keratinocytes supports a model where E3 protein suppresses cytokines via the RLR pathway[140]. However, it is still unclear whether RIG-I and MDA5 operate independently or collectively to initiate IFNβ secretion. Since an E3L-knockout virus is highly attenuated in a mouse model, the *in vivo* analysis of E3 protein function is difficult to investigate. Data available suggest that E3 protein may promote replication through inhibition of PKR and RNAseL pathways[141]. More *in vivo* studies will be needed for a complete understanding of E3 protein's role in VACV replication, especially regarding the function of the N-terminal domain.

3 Capping of mRNA

3.1 The eukaryotic mRNA cap

A significant post-transcriptional modification of RNA is the addition of an m^7G moiety linked via a 5'-5' triphosphate bridge to the first nucleotide of the transcript[142]. This structure is known as the mRNA 5' cap and is essential for mRNA stability, splicing, nuclear export and recognition by translation initiation complex[143,144]. The cap also provides eukaryotic cells a

means to discriminate between self and foreign RNAs, which are potent activators of IFNs[145,146]. The removal of the 5' cap by decapping enzymes allows degradation by 5'-3' exonucleases (such as XRN1). This process is employed by eukaryotic cells as a means of regulating mRNA transcript lifespan/turnover.

Cellular mRNAs are modified co-transcriptionally within the nucleus. Cap-synthesizing enzymes are recruited during transcription pausing, which occurs when the transcript is roughly 20-25 nucleotides long. Capping of transcripts occurs using three enzymatic reactions. The first reaction involves the removal of the γ-phosphate from the 5' end of the transcript by an RNA 5'-triphosphatase (TPase) to form 5'-diphosphate RNA (pp-RNA). The second reaction involves a 'ping-pong' mechanism by which a guanyltransferase (GTase) forms a covalent link with a guanosine monophosphate molecule, which is then transferred to the 5'-diphosphate RNA to form 5' G-ppp-RNA. The final reaction involves the methylation of the guanosine moiety by an S-adenyl-L-methionine (AdoMet) (guanine N7)-methyltransferase (MTase) to form m^7G-ppp-RNA. A summary of these enzymatic reactions can be found in Figure 1.10. These three reactions produce the cap-0 structure, which is the minimal chemical structure required for recognition by the translation initiation complex[147]. Further methylation reactions can occur on the first and second nucleotides downstream of the cap triphosphate bridge. These reactions are catalyzed by (nucleoside-2'-O)-methyltransferases (2'-OMTase) and comprise the cap-1 and cap-2 structures, respectively. Cap-1 and cap-2 structures are important regulators of antiviral responses involving stimulation of RIG-I and MDA5 pathways. The importance of the mRNA 5' cap structure is underscored by the co-evolution of similar processes in viruses to 'mask' their own transcripts and allow efficient translation of viral products (to be discussed below). Translation of cellular mRNAs is dependent on recognition of proper 5' cap structure and recruitment of ribosomal subunits. Initiation of translation in eukaryotes occurs through the formation of an 80S ribosome by a number of proteins named eukaryotic initiation factors (eIFs). The current model of eukaryotic translation divides the process into eight stages, which are depicted in Figure 1.11 (see ref *148* for a more detailed review)[148]. Firstly, eIF2 bound to GTP associates with the initiator codon, Met-$tRNA^{Met}_i$ to form the ternary complex. This complex then recruits the 40S subunit and factors eIF1, eIF1A, eIF3, and eIF5 for the formation of the 43S preinitiation complex. Meanwhile, the eIF4F complex (which comprises the cap-binding protein eIF4E and associated factors eIF4G and eIF4A) interacts with eIF4B to bind the 5' end of the mRNA transcript and unwind the 5'UTR in an ATP-dependent manner. The

unwinding of the 5' end allows the 43S preinitiation complex to be loaded onto the transcript and scanning commences in a 5' to 3' direction. Upon recognition of an AUG initiation codon, the scanning complex undergoes a conformational change resulting in the displacement of factor eIF1 and hydrolysis of eIF2-bound GTP by eIF5. This complex is known as the 48S initiation complex. Following hydrolysis, eIF5B mediates the dissociation of eIF2-GDP, eIF1, eIF3, eIF4B, eIF4F and eIF5. The 60S subunit is also recruited and joins to the 48S complex. Finally, an elongation-competent 80S ribosome is assembled from an additional GTP hydrolysis reaction by eIF5B and subsequent release of factors eIF1A and GDP-bound eIF5B. Following termination, ribosomal subunits are recycled for another round of translation.

Regulation of translation occurs by one of two methods (see ref *148* for a more detailed review)[148]. The first method involves regulating the activity of eIFs, mainly eIF2 and eIF4F, by phosphorylation. Phosphorylation of the alpha subunit of eIF2 prevents its recycling as phospho-eIF2α tightly associates with eIF2B and inhibits the exchange of GDP for GTP, preventing subsequent rounds of translation initiation from occurring. There are four kinases capable of phosphorylating eIF2α, EIF2AK1, PERK, Gcn2 and PKR. PKR is a crucial kinase involved in the antiviral response. The second method of regulation involves selectively targeting mRNA transcripts by sequence-specific RNA-binding proteins or microRNAs. This form of regulation involves preventing the formation/binding of initiation complexes to the 5’ end of the transcript or targets the mRNA for degradation. Viruses have evolved numerous strategies to exploit these processes for viral protein synthesis and virion production (to be discussed below).

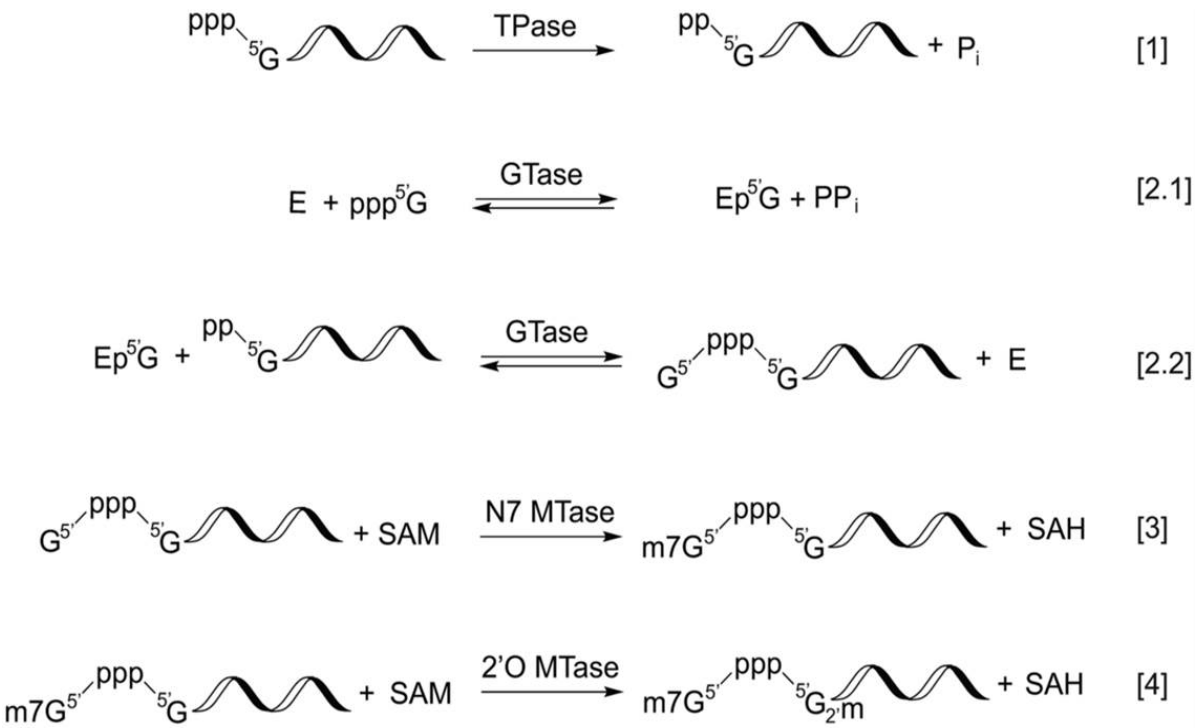


Figure 1.10 Enzymatic reactions required for mRNA capping.
The 5' end of RNA transcripts is modified by 4 enzymatic reactions. 1) An RNA trisphophatase (TPase) removes the gamma-phosphate from the 5' end, generating a 5' diphosphate and inorganic phosphoate (P_i). 2) A guanylyltransferase (GTase) forms a covalent link with a phosphogaunosine ($Ep^{5'}G$) by converting a GTP molecule to GMP and then transfers the phosphogaunosine molecule to the 5' end of disphosphate RNA forming a 5'-5' triphosphate bridge. 3) The guanine-N7 methyltransferase (N7 MTase) adds a methyl group from S-adenosylmethionine (SAM) to the N7 amine of the 5' guanosine residue. This forms the cap-0 structure. 4) The 2'O MTase modifies the 2'O of the first ribose to generate the cap-1 structure. Reproduced with permission from Oxford University Press. (From Ramanathan, A and Robb, B. mRNA capping: biological functions and applications. *Nucleic Acids Res* 2016; 44:7511-7526.)

3.2 Innate immune responses to foreign RNA structures (RIG-I and MDA5) and virus evasion strategies

Pathogenic invasions from viruses and bacteria have compelled eukaryotes to establish mechanisms for their detection. Innate immunity is the first to encounter infiltrating organisms and rely on the recognition of PAMPs, such as foreign nucleic acids[149]. PRRs belonging to the NLR and RLR families are responsible for the detection of nucleic acids in the cytoplasm of host cells[150,151]. These receptors trigger signalling cascades that culminate in type I IFN, IL-1 and pro-inflammatory cytokine production to establish an antiviral state and prevent further spread of the virus[152,153].

RIG-I and MDA5 are important infection sensors and discriminate self and non-self RNAs based on 5' end post-transcriptional modifications[154]. Originally, evidence suggested that

RIG-I was responsible for recognizing 5’ triphosphate RNAs[155], while MDA5 recognized both dsRNA and ssRNA carrying cap-0 modifications[154,156]. It was inferred that methylated RNA does not activate RIG-I as it would create a steric conflict in the nucleic acid binding cleft of the CTD domain. This domain in MDA5 is structurally similar to RIG-I, however, sequencing revealed that the amino acids involved in 5' end recognition are different MDA5 and RIG-I.

A more recent crystallography study has shown that cap-0 structures are also recognized by RIG-I[157]. Devarker *et al*. (2016) demonstrated that 5’-triphosphate RNA and cap-0 RNA bound to RIG-I with nearly identical affinities, did not alter the overall structure of the helicase-fold within the binding domain and activated RIG-I signalling to similar extents. Cap-0 and 5’-triphosphate ssRNAs were shown to be signalling inactive as they could not interact with RIG-I. Therefore, cap-1 formation through 2’O-methylation of the first nucleotide is a critical step for RIG-I evasion. Devarkar *et al.* (2016) mapped the 2’O-methylation sensor of RIG-I to residue H380[158]. Mutating this residue results in high binding affinity of RIG-I with 2'O-methylated dsRNAs and significant increases in ATPase activity of RIG-I. In order to evade RIG-I and MDA5 detection, viruses have evolved a number of unique mechanisms to cap viral RNA transcripts.

3.3 Viral strategies for mRNA capping

As obligate parasites, viruses rely heavily on host proteins for processes, such as translation, for virion production. During infections, viruses often alter mRNA translation processes to favour the production of viral products while promoting the decay of host transcripts. The 5' mRNA cap is also important for recruitment of the 40S ribosomal subunit and efficient translation of transcripts. Therefore, having viral mRNA transcripts with a 5’ cap is critical for virus replication. Furthermore, this structure is vital to pathogenesis as is provides the ability to circumvent antiviral response pathways (to be discussed below). Viruses have evolved different capping strategies, thereby highlighting the importance of the mRNA cap.

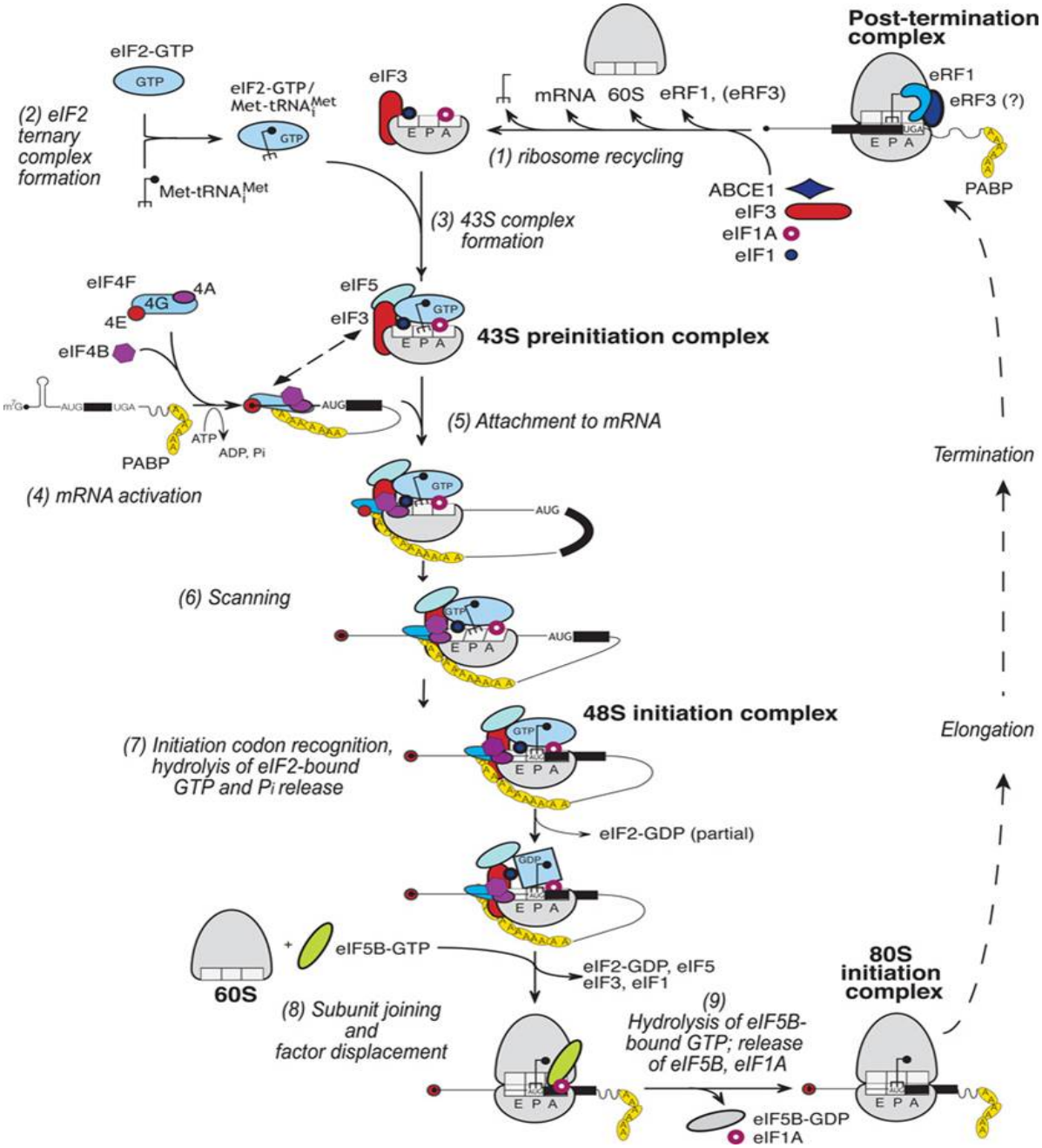


Figure 1.11 Schematic of eukaryotic translation initiation.
Translation initiation is divided into eight stages (2-9), which follow (1) recycling of 80S ribosomal subunits. (2) formation of eIF2 ternary complex, which is comprised of eIF2-GTP/Met-tRNA$^{Met}_i$. (3) formation of 43S preinitiation complex, which is comprised of a 40S subunit, eIF1, eIF1A, eIF3, eIF2-GTP/Met-tRNA$^{Met}_i$ and eIF5. (4) mRNA activation, where the 5' end (near the mRNA cap) is unwound by eIF4F and eIF4B in an ATP-dependent manner. (5) 43S complex attaches to the unwound mRNA region. (6) 43S complex scans the 5'UTR. (7) 48S initiation complex is formed upon recognition of the initiation codon, complex is switched to a 'closed' conformation which displaces eIF1, thereby allowing eIF5-mediated hydrolysis of eIF2-bound GTP. (8) 60S subunit is recruited and joins the 48S complex, displacing eIF2-GDP, eIF1, eIF3, eIF4B, eIF4F and eIF5 (mediated by eIF5B). (9) 80S initiation complex formed and release of eIF5B-GDP and eIF1A. Elongation occurs and upon termination the ribosomal subunits separate and are recycled for a following round of transcription. Reproduced with permission from Springer Nature. (From Jackson et al. The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat Rev Mol Cell Biol* 2010; 11:113-127.)

Generally, virus capping mechanisms can be grouped into conventional and unconventional categories. DNA viruses that commandeer the hosts' RNA polymerase also use cellular capping enzymes[159]. However, many viruses encode their own capping machinery and will cap mRNA transcripts using the same conventional mechanism as its host: triphosphate removal (TPase) followed by guanylation (GTase) and methylation (MTase and 2'-OMTase). These enzymes can vary between virus families, with one or more proteins responsible for the reactions necessary to form an operational 5' cap. For example, dsDNA viruses from *Poxviridae* encode three proteins for cap-1 synthesis (the D1-D12 heterodimer and J3 protein)[160–162]. These proteins catalyze mRNA capping in the cytoplasm of infected cells. On the other hand, dsRNA viruses from *Reoviridae* use structural proteins on an inner capsid in an 'assembly line' mechanism. These enzymes contain the four domains necessary for cap-1 synthesis and the resulting mRNA is released into the cytoplasm of infected cells[163,164].

Some viruses have evolved unique mechanisms for mRNA capping. For example, the family multimegavirale does not encode any capping machinery. Instead, these viruses evolved a cap-snatching pathway where 5' caps are stolen from host mRNAs and used to prime viral mRNA synthesis[165–167]. Nonetheless, the cap structures produced by these unconventional capping mechanisms are indistinguishable from the host. Convergence to a uniform cap structure underscores the selective pressures to maintain such a structure.

i. Capping enzyme of VACV

VACV encodes its own version of a capping enzyme and synthesizes the 5' cap using a conventional (TPase, GTase, MTase) approach. The capping enzyme of VACV is a heterodimer comprised of a large and small subunit encoded by the D1R and D12L genes, respectively. This enzyme is responsible for the three enzymatic reactions for cap-0 synthesis[168,169]. Additional methylation for cap-1 formation is performed by VACV J3 protein, which also acts as the viruses poly(A) polymerase[162].

The active sites for the three reactions of the capping enzyme are housed solely within the large subunit and have been charted in detail (see ref *170* for a detailed review)[170]. The N-terminal region of D1 (amino acids 1-545) contains the TPase and GTase domains[171,172]. Structural studies of this domain revealed the TPase segment belongs to the family of triphosphate tunnel metalloenzymes[170], which hydrolyze nucleotide triphosphates in the presence of Mn^{2+} or Co^{2+} [173,174]. A similar enzyme family is also used by other DNA viruses[173,175–177], mimiviruses and fungal TPases[173,175] . VACV capping enzyme GTase segment consists of a

nucleotidyltransferase domain followed by an oligonucleotide-binding domain. Interestingly, this module is analogous to DNA ligases, as well as cellular and other viral capping enzymes[178].

The C-terminal domain of D1 is responsible for guanine-N7 methylation (MTase domain)[179,180]. This segment sparked initial interest in functional and structural investigations[160,181] and has been characterized as a class I AdoMet dependent MTase[170]. Although the active site for guanine-N7 methylation resides in the C-terminal MTase domain of the capping enzyme, Zheng and Shuman (2008) discovered a 'lid peptide' in the N-terminus[182]. Using crystallography, the authors demonstrated that amino acids 545-563 close over the MTase active site (amino acids 498-844)[183] and use Van der Waals contacts to stabilize the transition state during the methylation procedure[182].

Another interesting feature of the VACV capping enzyme is the presence of a small subunit, the D12 protein. D12 is a unique feature of poxviruses as there have been no discernible homologs discovered in any eukaryotic proteomes to date. The small subunit itself was reported to resemble the 2'-OMTase of RNA polymerase from reovirus and flavivirus[160]. However, D12 lacks the binding site for AdoMet and therefore is unable to perform the reaction necessary for methylation. Instead, D12 functions as a regulatory subunit in the VACV capping enzyme, by allosterically stimulating the MTase activity of the D1 subunit[184–186]. In addition to MTase activity, heterodimerization of the D1 and D12 subunits is also required for the other known functions of VACV capping enzyme.

ii. Alternative functions of D1/D12 heterodimer

The majority of studies involving VACV capping enzyme have focused on its role in 5' capping and mapping of the active sites for the three enzymatic reactions culminating in cap-0 formation. The D1-D12 heterodimer also has critical roles in the accurate transcription termination of early genes[186–188] and transcription initiation of intermediate genes[189]. Poxvirus transcription events occur in 3 waves: early, intermediate and late[42]. To achieve this, VACV RNA polymerase has two distinct forms. The RNA polymerase consists of 8 subunits, and an additional subunit (H4 protein) is necessary for the recognition of the unique promoter sequence for early genes[42]. This additional factor is absent in the RNA polymerase that synthesizes intermediate and late transcripts.

VACV early transcripts also contain a unique termination sequence (U5NU), thereby creating homogeneous 3' ends. Although both subunits are required for early transcription termination activity[186], it has been demonstrated that the TPase domain of D1 specifically

recognizes the termination sequence[190]. Notably, the TPase, GTase, and MTase functions of the capping enzyme are irrelevant in early termination activity[186]. Upon recognition of the termination sequence, VACV NPHI (product of the D11L gene) interacts with the early RNA polymerase via contacts with the H4 subunit and catalyzes the termination reaction[191–194].

Intermediate and late transcripts are synthesized after viral DNA replication and also have stage-specific promoters. However, they lack defined termination sequences, thereby resulting in heterogeneous 3' ends. The VACV D1/D12 heterodimer is one of three factors required for initiation of intermediate gene transcription[189]. Similarly, capping functionality is not required for initiation events. However, both subunits must be present[183,195]. The mechanism for initiation of intermediate gene transcription is not well understood. Investigations have suggested that the D1/D12 heterodimer must form a stable complex with the RNA polymerase prior to transcription initiation[170,186]. The other factor involved, termed VITF-1, was identified as VACV E4 protein[196], which also acts as an RNA polymerase subunit. Interestingly, E4 protein also displays homology to the eukaryotic transcription elongation factors SII (TFSII).

Although the catalytic sites have yet to be identified, recent biochemical data from a temperature-sensitive VACV mutant has demonstrated that the G705 residue of the D1 subunit is critical for both transcription termination and initiation activities[197]. This mutant demonstrated increases in transcriptional readthrough of early genes and lowered levels of intermediate genes. No change was observed in the products from late genes. Additionally, this mutant displayed compromised GTase and MTase activities. The authors speculate that the mutation results in a conformational change in the MTase domain that also alters the GTase domain. They also suggest that the transcription termination and initiation activities involve an interaction between the GTase and/or MTase domain of the capping enzyme with the viral RNA polymerase.

4 Gaps in Knowledge and Study Rationale

4.1 E3 ortholog recombinant viruses – SPPV034 does not rescue function

Current work in our lab has been focused on the biological function of E3 family proteins. One study from our lab investigated the E3 protein orthologs from other *chordopoxviruses*[132]. Recombinant viruses were generated in which an E3 ortholog was inserted into the E3L locus. Results from this study indicated that the ortholog from sheeppox virus, SPPV034, was the only ortholog unable to rescue E3 protein functionality. Interestingly, the SPPV034 protein retained the ability to bind dsRNA as assessed by a pulldown assay using poly

I:C. These results indicate that SPPV034 is unable to function in an unidentified role/mechanism for virus replication to occur.

An additional study investigated the differences between SPPV034 and E3 protein functionalities. Chimeric constructs in which the N-terminal domain of SPPV034 was fused with the C-terminal domain of E3 and the N-terminal domain of E3 was fused with SPPV034 C-terminal domain were generated and inserted into the E3 locus. Interestingly, the SPPV034 N-terminus impaired the function of the E3 C-terminal domain as this recombinant virus demonstrated lower replication than full-length E3 protein (*Varga and Cao; manuscript in preparation*). On the other hand, the E3 N-terminus enhanced the function of the SPPV034 C-terminus as this recombinant virus replicated better than the full-length SPPV034 protein. Both viruses retained the ability to bind dsRNA as assessed by a pulldown using poly I:C. These data indicate that the N-terminus of E3 family proteins plays an important regulatory role in the proper functioning of the protein. Interestingly, these viruses replicated to significant titres but were unable to fully inhibit activation of PKR as with the wild-type E3 protein.

4.2 E3 mutagenesis – dsRNA binding and E3 biological function not linked

Another study further investigated residues in the C-terminal region of the E3 protein identified as critical for protein function *in vitro*. Residues in the E3 protein were mutated to alanine and inserted into the E3 locus. Results uncovered nine mutations that caused defects the dsRNA binding ability of the E3 protein and but these mutants retained host range function (replicated to various degrees in non-permissive HeLa cells)[133]. These mutants had varying effects on host antiviral responses, including apoptosis, inhibition of PKR activation, cytokine suppression. Interestingly, it was demonstrated that the mutant E3 proteins interact with PKR as assessed by a co-immunoprecipitation assay. These data indicate a novel mechanism (one that does not require dsRNA binding) for the biological function of E3 family proteins.

4.3 EMS mutagenesis uncovers link between capping enzyme and E3 family proteins

The current study used EMS mutagenesis to investigate a possible viral cofactor necessary for the biological function of the E3 protein. Potential candidates were all involved in a viral RNA processing mechanism, however, two mutations in the capping enzyme (comprised of the D1 and D12 heterodimer) were the only ones capable of independently rescuing replication of the SPPV034 recombinant virus. This enzyme is also involved in vaccinia early

gene transcription termination and intermediate gene transcription initiation (see above for a more detailed review).

E3 family proteins play crucial roles in preventing host innate antiviral activities activated by virus dsRNA (a significant PAMP involved in the activation of the host antiviral responses). The mRNA cap of viral transcripts is also critical in the activation of RLR signalling cascades and antiviral responses. The D1-D12 heterodimers role in early gene transcription termination can be speculated as also having a crucial role in regulating dsRNA levels early in infection. An E3-knockout vaccinia virus demonstrates aborted replication, specifically through blocked intermediate and late protein translation. The SPPV034 recombinant virus also demonstrates a similar phenotype, in that the synthesis of its intermediate and late protein are also blocked. Therefore, it appears the D1-D12 heterodimer coordinates functions with E3 family proteins for an effective shift from early to intermediate gene transcription.

4.4 Hypotheses

I hypothesize that the E3 ortholog, SPPV034, is unable to interact with a vaccinia virus cofactor and that this interaction is critical for the proper functioning of E3 family proteins.

I hypothesize that the D1-D12 heterodimer is the cofactor for proper E3 family protein functionality and fosters effective host immune modulation.

4.5 Objectives

i. The first objective of this study is to characterize the immunomodulatory functions of the SPPV034 recombinant virus after introduction of a mutation in the D1 or D12 subunit of the capping enzyme.
ii. The second objective of this study is to analyze the differences in dsRNA production of the SPPV034 recombinant virus after introduction of a mutation in the D1 or D12 subunit of the capping enzyme.
iii. The third objective of this study is to investigate if E3 family proteins are involved in virus mRNA translation initiation.

Chapter Two: Materials and Methods

1 Cells and Viruses

Baby hamster kidney (BHK21) and human cervical carcinoma epithelial (HeLa) were purchased from ATCC. Human cervical carcinoma epithelial PKR-knockout (HeLa PKR knockout) were created using CRISPR-Cas9 and generously provided by Dr. Jim Smiley, Unversity of Alberta. Adenocarcinomic human alveolar basal epithelial cells (A549) PKR knockout, RNAseL knockout and double (PKR and RNAseL) knockout cells were generated using CRISPR-Cas9 and provided by Dr. Bernard Moss, NIH. All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-glutamine (Gibco) at 37°C and 5% CO_2. Passaging of cells occured once they reached 80-100% confluency. Briefly, culture medium (as described above) was removed, cells were washed with phosphate buffered saline (PBS) and dissociated using 0.05% Trysin-EDTA (Gibco) at 37°C. Approximately, 5-15% of cells were used to seed subsequent passage.

All viruses used in this study are based on vaccinia virus Western Reserve strain (WR-V119) (ATCC). Viral infections were conducted using DMEM supplemented with 2% fetal bovine serum and 1% penicillin-streptomycin-glutamine (Gibco).

2 Reagents

Protein loading buffer – 100mM Tris-HCl (pH 8.0), 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200nM 2-mercaptoethanol. Made at 2X concentration and diluted with water.

Non-denaturing lysis buffer – 20mM Tris HCl (pH8.0), 137mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, supplemented with 10U/mL Ribolock RNase inhibitor (Thermo Scientific) and 1 Protease Inhibitor Cocktail tablet (Roche).

Plaque assay agar overlay – 2X minimum essential medium (MEM) (Gibco), 0.45% sodium bicarbonate, 2% FBS, 1% penicillin-streptomycin-glutamine. Made at 2X concentration and diluted 1:1 with 2% Ultrapure low melting point agarose (Invitrogen).

Antibodies – all antibodies used in this study are listed in Table A 3.

3 Generation, whole genome sequencing and selection of mutant viruses

The recombinant vaccinia virus expressing the sheeppox virus E3L ortholog, SPPV034L, was previously described (*Varga and Cao; manuscript in preparation*) and termed SPPV034. This recombinant was used as the parental strain for ethyl-methanesulfonate (EMS) mutagenesis. A schematic of the workflow for the generation of the SPPV034 recombinant virus is depicted in Figure A 1.

BHK21 cells were infected with SSPV034 recombinant virus for 2 hours and treated with 200μg EMS. Media was replaced after 6 hours. Virus was collected the following day, passaged in HeLa cells 5 times, 12 plaques were selected and their genomes were sequenced at the National Microbiology Laboratory's DNA Core. Sequencing identified 5 non-synonymous mutations (Table 3.1). Primers were used to induce point mutations identified in candidate gene sequences. These genes were individually transfected into the original SPPV034 recombinant virus, passaged in HeLa cells 5 times and viable viruses were selected for further analysis. Two candidates were capable of individually rescuing replication after subsequent passaging in HeLa cells. These mutations were a G801S substitution in D1R and an S131N substitution in D12L. These genes correspond to the large and small subunits of the mRNA capping enzyme of vaccinia virus, respectively. These mutant viruses were termed SPPV034/D1-G801S and SPPV034/D12-S131N, respectively. A schematic of the workflow for the EMS mutagenesis for the insertion of the mutation in the D1 or D12 subunit is depicted in Figure A 2.

The mutant viruses were then used to create knock-out control viruses. A previously designed vector[132] (pWRΔE3L/EGFP) was used to remove the SPPV034L gene from mutant virus's SPPV034/D1-G801S and SPPV034/D12-S131N. BHK21 cells were infected with parental virus (either SPPV034/D1-G801S or SPPV034/D12-S131N) and transfected with 1 μg of pWRΔE3L/EGFP plasmid. Virus exhibiting green fluorescent protein only, indicating a double crossover event, were selected using UV microscopy and purified using 3 rounds of plaque purification. The resulting knock-out viruses were termed vvΔE3/D1-G801S and vvΔE3/D12-S131N, respectively. A schematic of the workflow for the removal of the SPPV034L gene is depicted in Figure A 3.

Constructs were sent to the National Microbiology Laboratory DNA Core for a second round of whole genome sequencing to verify that samples contained the correct mutation from original screening and that no other mutations had occurred. A summary of all viruses used in this study are listed in Table A 1. Schematics of all virus genomes and plaque growth in HeLa and BHK21 cells are depicted in

Figure A 4.

4 Virus replication assays

Single Step Growth Kinetics

Cells infected at multiplicity of infection (MOI) 5 and incubated at 37°C + 5% CO_2 for 1 hour. Virus was removed, monolayers washed with phosphate buffered saline (PBS) 2 times and 1 millilitre (mL) of fresh virus medium was added. Virus was collected at 5 and 24 hours post infection (hpi). Virus was released using 3 cycles of freeze and thaw, and supernatant was transferred to fresh tubes. Plaque assays were performed in BHK21 cells. BHK21 cells in 12 well plates were infected at appropriate dilutions to achieve >100 plaques per well and incubated at 37°C + 5% CO_2 for 1 hour. Medium was removed, 1 mL of 1X agar overlay was added and plates were placed back in the incubator. Plaques were counted at 48 hpi using a UV microscope at 10 times magnification. Plaque assays were performed in triplicate and the average number of plaque forming units per mL (pfu/mL) was calculated. The virus titre difference between the virus preparations collected at 5 hpi and 24 hpi represent virus growth.

Multiple Step Growth Kinetics

Cells were infected at MOI 0.1 and incubated at 37°C + 5% CO_2 for 1 hour. Virus was removed, monolayers washed with phosphate buffered saline (PBS) 2 times and 1 mL of fresh virus medium was added. Virus was collected at 5, 24, 48, 72 and 96 hpi. Virus was released using 3 cycles of freeze and thaw, and supernatant was transferred to fresh tubes. Plaque assays were performed in BHK21 cells as described above.

A549 plaque formation

A549 cell monolayers were infected in serial dilution in 48 well plates. Plaque formation was monitored using UV microscopy at 24 and 48 hpi.

5 Western blotting

Cell monolayers in a 12-well plate were infected at MOI indicated in the results section and harvested at time points as described in the figure legends. Cells were lysed using 200 microlitres (µL) of protein loading buffer and homogenized by passing through a Qiashredder (Qiagen) at 12,000 rpm for 2 minutes. Aliquots of 20 µL were boiled at 99°C for 5 minutes. Proteins were separated using precast NuPAGE 4-12% bis-tris 20 well gels (Invitrogen) and 1X MOPS running buffer. Proteins were transferred to Immun-Blot PVDF membrane (BioRad)

using a Trans-Blot turbo apparatus (Bio-Rad). Transfer buffer consisted of 1X Trans-Blot Turbo transfer buffer (BioRad) and 19% ethanol. Membranes were blocked in a 1:1 solution of Odyssey blocking buffer and phosphate buffered saline (PBS) (LiCor) at room temperature for 4 hours. Membranes were incubated with the indicated antibodies in 1:1 solution of Odyssey blocking buffer and PBS+0.1% Tween-20 (PBST) at 4°C overnight. The following day membranes were washed for 5 minutes, 3 times with PBST. Membranes were incubated with secondary antibodies, labeled with IRDye near-infrared fluorescent dye (LiCor), in a 1:1 solution of Odyssey blocking buffer and PBST supplemented with 0.01% SDS at room temperature for 1 hour. A list of antibodies used in this study can be found in Table A 3. Membranes were then washed for 5 minutes, 4 times with PBST and washed 2 more times with PBS to remove any detergent. Proteins were detected using a LiCor Odyssey near-infrared scanner.

6 Quantitative Real-time PCR (RT-qPCR)

HeLa cells were infected at MOI 5 and RNA was extracted at 8 hpi using RNEasy Mini kit (Qiagen). DNA was digested using TURBO DNA-free kit (Invitrogen) according to manufacturer's instructions. Total RNA concentration was determined by measuring absorbance at 260 nanometers (nm) using a NanoDrop 2000 spectrophotometer (Thermo Scientific). 1 μg of RNA was reverse transcribed using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific) and diluted using 80 μL of nuclease free water (Invitrogen).

Quantitative real-time PCR (RT-qPCR) was performed using TaqMan Gene Expression Master Mix in MicroAmp Fast Optical 96-well plates (Applied Biosystems) on a StepOnePlus real time PCR machine (Applied Biosystems). Sequences for real time PCR primers and probes (Applied Biosystems) are listed in Table A 2. The real-time PCR cycle consisted of an initial step of 50°C for 2 minutes, followed by 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. To account for total RNA loading, expression of actin was used as an internal normalization control. All reactions were performed in triplicate.

7 Immunofluorescent microscopy

Four chambered slides containing HeLa or HeLa PKR-knockout cells were infected at MOI 5 and incubated at 37°C. At 5 and 12 hpi cells were washed twice with PBS and fixed using a 3% para-formalin/PBS solution for 10 minutes at room temperature. Cells were washed for 3

minutes, 3 times with PBS and permeabilized using a 0.2% Triton X-100/PBS solution for 5 minutes at room temperature with gentle rotation. Cells were washed for 1 minute, 3 times with PBS and blocked using a 2% FBS, 2% BSA/PBS solution at room temperature for 1 hour. Cells were then incubated with the primary antibody, diluted 1/200 in blocking buffer, at room temperature for 2 hours. Cells were washed 3 times with PBS for 3 minutes and incubated with secondary antibody, diluted 1/1000 in blocking buffer, at room temperature for 1 hour. Cells were washed 3 more times with PBS for 3 minutes each. The chambers were removed from the slides and rinsed with water. Prolong antifade with DAPI (Invitrogen) was added to each slide, covered with a coverslip and incubated at room temperature in the dark overnight. Slides were imaged using a confocal microscope (purchased by Dr. Stephanie Booth, National Microbiology Laboratory) (Zeiss) at 63 times magnification. Image analysis was performed using ZEN Black (Zeiss) and Fiji (ImageJ) software.

8 Dot blot assay and RNA quantification

Confluent BHK21, HeLa, and HeLa PKR knockout cells were infected at MOI 5 and RNA was extracted at 5 and 12 hpi using a RNEasy Mini kit (Qiagen). Any leftover DNA was digested using TURBO DNA-free kit (Invitrogen) according to manufacturer's instructions. RNA concentration was determined by measuring absorbance at 260nm using a Nanodrop 2000 spectrophotometer (Thermo Scientific). Confluent BHK21, HeLa, and HeLa PKR knockout cells were treated with cytosine arabinoside (AraC) (50µg/mL) (Sigma) and infected at MOI 5. RNA was extracted at 12 hpi and processed as above.

Nitrocellulose membranes (BioRad) were soaked in 6X SSC buffer (saline sodium citrate) for 10 minutes. Membranes were loaded into a Bio-Dot apparatus (BioRad) and 100 µL of 6X SSC buffer were loaded into the wells. RNA concentrations of 1, 3, and 5 µg in 60 µL of 6X SSC buffer were loaded, in triplicate, into the apparatus. Membranes were incubated at room temperature for 1.5 hours and a vacuum was applied to transfer any remaining liquid through the membrane. Membranes were blocked in a 1:1 solution of Odyssey blocking buffer (LiCor) and PBS at room temperature for 4 hours. Membranes were incubated overnight at 4°C with anti-dsRNA (J2) antibody (1:1000) (SCICONS), diluted in a 1:1 solution of Odyssey blocking buffer and PBS supplemented with 0.1% Tween-20 (PBST). The following day, membranes were washed 3 times in PBST for 5 minutes. Membranes were incubated at room temperature for 1

hour with IRDye 680LT Goat anti mouse antibody (1:10000) (LiCor), diluted in a 1:1 solution of Odyssey blocking buffer and PBST supplemented with 0.01% SDS. Membranes were washed 4 times in PBST and washed 2 more times with PBS to remove any detergent. dsRNA was detected using a LiCor Odyssey near-infrared scanner.

RNA quantification was performed using Image Studio Lite software (Ver 5.2). dsRNA antibody signal was averaged among triplicates and normalized to uninfected cells to give dsRNA production values.

9 Isolation and transfection of viral RNA

BHK21 cells were infected at MOI 5 and RNA was harvested at 5 and 12 hpi. RNA was extracted using the RNEasy Mini kit (Qiagen) and DNA was digested using the TURBO DNA-free kit (Invitrogen) according to manufacturer's protocols. Total RNA concentration was determined by measuring absorbance at 260nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific). To prevent multiple freeze and thaws, 15 μL aliquots were created and stored at -80°C. HeLa cells, at 80-90% confluency, were transfected with 3 μg of total RNA. Cells were transfected in duplicate so that both RNA and protein could be harvested. Uninfected BHK21 cellular RNA served as a mock transfection control.

Protein was harvested at 8 hpi by adding 200 μL of 1X protein loading buffer and lysates were homogenized by passing through a Qiashredder (Qiagen) at 12,000 rpm for 2 minutes. Immunomodulation of cells by RNA products was analyzed using western blotting as described above.

RNA was harvested at 8 hpi using the RNEasy Mini kit (Qiagen). DNA was digested using TURBO DNA-free kit (Invitrogen) and 1 μg of RNA was reverse transcribed using the Maxima RT cDNA synthesis kit (Invitrogen), followed by dilution with 80 μL of nuclease free water (Invitrogen). Changes in the cytokine profile of the cells was analyzed using qPCR as described above.

10 Co-immunoprecipitation assay

Co-immunoprecipitation of host ribosomal factors with vvE3 protein

HeLa cells were infected at MOI 1 with a biotinylation recognition signal flanked by two hexahistidine sequences (HBH)-tagged E3L-Rev virus (as described in section 3; using an HBH-tag sequence instead of the flag-tag) and incubated overnight. Monolayers were washed twice with 1mL cold PBS and lysed using 500 μL of non-denaturing lysis buffer and a freeze and thaw. Cell debris was removed by centrifugation at 1500xg at 4°C for 10 minutes and 100 μL was removed as pre-pulldown samples. Supernatant was incubated with pre-blocked Dynabeads MyOne Streptavidin T1 magnetic beads (Invitrogen) at room temperature for 2 hours. Beads were washed 4 times using 500 μL non-denaturing lysis buffer. Bound peptides were removed by addition 200 μL 1X protein loading buffer and boiling at 95°C for 15 minutes. Pre-pulldown samples were processed by addition of 100 μL 2X protein loading buffer. Protein interactions were investigated using western blotting as described above.

Binding of the m7-GTP mRNA cap

HeLa PKR-knockout cells were infected with the indicated viruses at MOI 1 and incubate overnight. Aminophenyl-7-methyl-guanosine 5'-triphosphate (m7-GTP) cross linked agarose beads (Creative Biomart) were blocked in 2% FBS for 2 hours, washed 2 times with PBS and washed 2 more times with non-denaturing lysis buffer. Monolayers were washed, lysed and incubated with m7-GTP beads as described above. Protein-mRNA cap interactions were investigated using western blotting as described above.

Chapter Three: Results

1 EMS mutagenesis uncovered the mRNA capping enzyme as an E3 protein cofactor

The E3 family proteins refer to E3 orthologs from all poxviruses. These proteins contain an N-terminal Z-DNA binding domain and a C-terminal dsRNA protein domain. E3 family proteins have been established as crucial determiners of poxvirus' host range. Deletion of E3 from vaccinia virus severely impacts vaccinia virus replication *in vivo* and *in vitro*[198,199].

Previous work investigating the host range function of E3 family proteins have yielded interesting results. An E3-knockout vaccinia virus has a restricted host range and is unable to replicate in HeLa cells. Sheeppox virus has a restricted host range and only replicates in OA3.Ts cells (a lamb testis cell line). Repeated efforts to delete the E3 ortholog (SPPV034) from wild-type sheeppox virus in OA3.Ts cell infections have failed, indicating that the gene is essential for the virus replication (*JXC unpublished data*). E3 protein is also required for vaccinia virus replication in OA3.Ts cells. E3 ortholog functionality has been studied extensively, and it was discovered that the E3 ortholog from sheeppox virus, SPPV034, is the only one not able to restore host range function[132] (*JXC unpublished data*). For a summary of the host range of these viruses as mentioned above see Table 3.1. Therefore, it was postulated that the function of E3 family proteins could be dependent on the presence of a virus-specific cofactor(s). This specific vaccinia virus cofactor could be unable to function in conjunction with the sheeppox virus E3 ortholog, SPPV034, resulting in the inability of the recombinant SPPV034 vaccinia virus to restore the host range function.

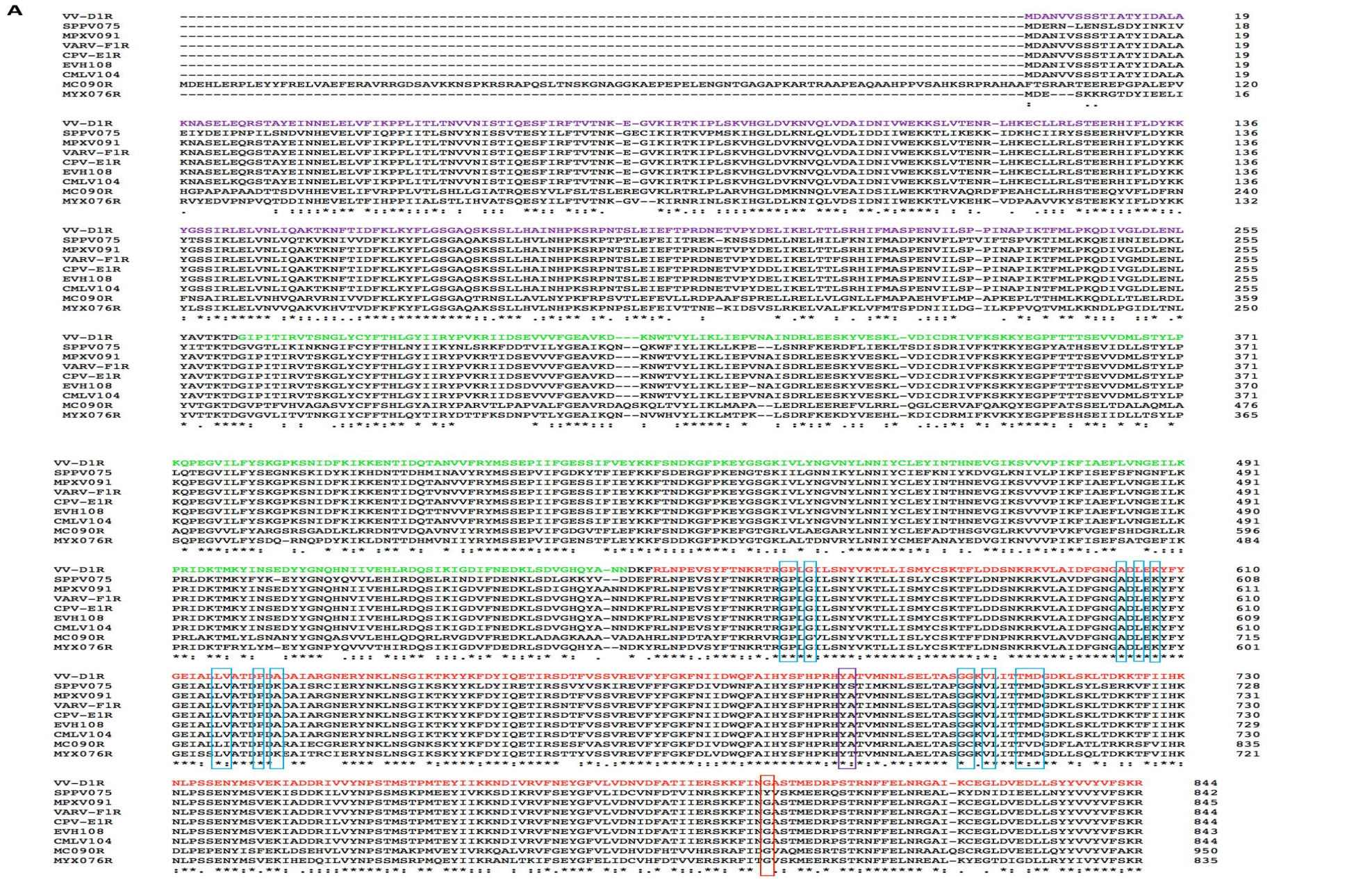
To identify potential cofactors of E3 family proteins, the recombinant SPPV034 vaccinia virus (SPPV034) was treated with an alkylating agent, ethylmethanesulfonate (EMS), to induce mispairing. EMS acts by randomly adding an oxygen molecule to a guanine residue, thereby creating O^6-methylguanine residues. These alkylated guanine residues are paired with thymine residues in subsequent rounds of DNA replication, resulting in GC→AT mutation[200]. The resulting viruses were screened for mutations that rescued replication in HeLa cells. Whole-genome sequencing (WGS) of selected mutant viruses capable of replicating in HeLa cells revealed 5 potential candidate proteins as cofactors for E3 family proteins (Table 3.1). These candidates included the F4, J3, D1, D11 and D12 proteins.

Table 3.1. Non-synonymous mutations identified after mutagenesis of the SPPV034 recombinant virus.

Gene	Position	BP Change	AA Change	Gene Product
F4L	30508	G→A	P215S	ribonucleotide reductase small subunit
J3R	80396	C→T	P254S	poly(A) polymerase small subunit
D1R	89142	G→A	G801S	large subunit; mRNA capping enzyme
D11L	105442	G→A	T57M	ATPase, nucleotide triphosphate phosphohydrolase-I, NPH-I
D12L	106118	C→T	S131N	small subunit; mRNA capping enzyme

From these candidates, single mutations were introduced into the genome of a recombinant vaccinia virus expressing SPPV034 using homologous recombination. These mutations corresponded to a single nucleotide substitution as identified above. The viruses which were capable of replicating in HeLa cells were selected for further analysis. Only 2 candidates were able to rescue replication of the recombinant virus after subsequent passaging in HeLa cells. A mutation in the D1R gene, resulting in a G801S substitution, and a mutation in the D12L gene, resulting in an S131N substitution, were able to independently restore host range function to the SPPV034 recombinant virus. The D1R gene encodes the vaccinia virus mRNA capping enzyme large subunit, while the D12R gene encodes the small subunit. The mutations identified do not occur in residues previously identified as necessary for methyltransferase activity or heterodimerization ability of the enzyme (Figure 3.1)[161,201].

The amino acid sequences of the mRNA capping enzyme of *Poxviridae* family members were aligned using Clustal Omega Software. The D1 orthologs of these poxviruses, including sheeppox virus (SPPV), monkeypox virus (MPX), variola virus (VARV), cowpox virus (CPV), ectromelia virus (EVH), camelpox virus (CMLV), molluscum contagiosum (MC), and myxoma virus (MYX), share approximately 64, 99, 99, 99, 99, 99, 57, and 65% sequence identity with vaccinia virus respectively (Figure 3.1A). The residues previously identified as critical for the methyltransferase activity and heterodimerization[161] of vv-D1 are highly conserved (highlighted in Figure 3.1A). Using the VACV sequence as a reference, changes in the sheeppox virus ortholog included an A to K substitution at residue 623 and an A to S substitution at 691.


A
VV-D1R
SPPV075
MPXV091
VARV-F1R
CPV-E1R
EVH108
CMLV104
MC090R
MYX076R

B

Figure 3.1. Alignment of *Poxviridae* family members amino acid sequences.
Amino acid sequences of the mRNA capping enzyme large (A) and small (B) subunits were downloaded from NCBI GenBank and aligned using Clustal Omega software. An asterisk (*) denotes a fully conserved residue, a semi-colon (:) denotes residues with strongly similar properties, and a period (.) denotes residues with weakly similar properties. Virus species: vaccinia virus (VACV), sheeppox virus (SPPV), monkeypox virus (MPXV), variola virus (VARV), cowpox virus (CPV), ectromelia virus (EVH), camelpox virus (CMLV), molluscum contagiosum (MC), myxoma virus (MYX; rabbitpox). (**A**) Alignment of the D1 subunit of the mRNA capping enzyme. Since the domains are known in VACV, these are highlighted by color. The GTPase domain is highlighted in purple, the TPase domain is highlighted in green and the MTase domain is highlighted in red. Residues critical for both the MTase activity of D1R and D1-D12 heterodimerization is surrounded in a blue box, while residues critical only for the MTase activity of D1R is surrounded by a purple box. The mutated residue is surrounded by a red box; mutagenesis of VACV D1R resulted in a G→S residue change. (**B**) Alignment of the D12 subunit of the mRNA capping enzyme. Residues critical only for the MTase activity of D1R is surrounded by a purple box, residues critical for both the MTase activity of D1R and D1-D12 heterodimerization are surrounded by a blue box, and residues critical only for D1-D12 heterodimerization are surrounded by a green box. The mutated residue is surrounded by a red box; mutagenesis of VACV D12L resulted in a S→N residue substitution.

Changes in the *molluscipoxvirus* molluscum contagiosum included a V to I substitution at residue 616, a G to C substitution at 705, and an M to V substitution at 712. Changes in the *lepripoxvirus* myxoma virus (rabbitpox) included an A to K substitution at residue 623 and an A to T substitution at 691. Interestingly, the mutated residue identified from EMS mutagenesis was conserved in all orthologs except SPPV. Instead, the sheeppox virus ortholog contained a G to Y substitution at residue 801.

The D12 orthologs of SPPV, MPX, VARV, CPV, EVH, CMLV, MC, and MYX share approximately 75, 99, 99.3, 99.7, 99.3, 99.3, 60, and 71% sequence identity with vaccinia virus, respectively (Figure 3.1B). The residues previously identified as critical for methyltransferase activity and heterodimerization[201] of vvD12 are also highly conserved (highlighted in colour in Figure 3.1B). Changes in the SPPV ortholog included an M to L substitution at residue 60. Changes in the MC ortholog included an R to K substitution at residue 50, an L to V substitution at 61, and a K to Q substitution at 244. Changes in the MYX ortholog included an R to K substitution at residue 50 and an M to L substitution at 60. Interestingly, the mutated residue derived from the EMS mutagenesis is conserved between vaccinia virus and sheeppox virus (both originally contain an S residue at position 131). MYX contained a non-conserved residue at this position and displayed an S to G substitution at residue 131.

2 Mutations in the vaccinia mRNA capping enzyme rescue replication of a recombinant vaccinia virus expressing the sheeppox virus E3L ortholog, SPPV034L

The replication capacity of the recombinant vaccinia viruses containing mutations in the mRNA capping enzyme was analyzed using high and low MOI infections in HeLa cells. For high MOI experiments, HeLa cells were infected at MOI 5 and virus was harvested at 5 and 24 hours post infection (hpi). Virus yield was determined using a plaque assay in BHK21 cells. For the low MOI experiments, HeLa cells were infected at MOI 0.1 and the virus was collected and titrated at 5, 24, 48, 72, and 96 hpi. As previously reported, the vvΔE3L and vvΔE3L/SPPV034 viruses were not capable of replicating in HeLa cells[132] (*Varga and Cao; manuscript in preparation*). The SPPV034/D1-G801S construct was able to rescue replication to the same degree as the vvE3L-Rev virus in high MOI infection (Figure 3.2A(i)), but to slightly less of a degree in a low MOI infection (Figure 3.2B(i)). The SPPV034/D12-S131N construct

demonstrated a moderate growth in both high and low MOI infections. However, this growth was more evident in the low MOI infection (Figure 3.2A(i) and B(i)). To confirm that the phenotype observed was a result of the combination of the mutation in the mRNA capping enzyme subunit and the presence of the E3 ortholog, SPPV034, the SPPV034L gene was deleted from the recombinant virus vvΔE3/SPPV034 bearing the D1R or D12L mutation. These viruses lack the SPPV034 protein but contain one of the mutations in the mRNA capping enzyme subunits. Both the vvΔE3/D1-G801S and vvΔE3/D12-S131N knockout viruses were unable to replicate in HeLa cells at either high or low MOI infections (Figure 3.2A(i) and B(i)).

A key feature of restricted vaccinia virus replication is blocked protein translation of intermediate and late transcripts. The expression of an early protein, vvD12, an intermediate protein, vvG8, and a late protein, vvD8, were analyzed using Western blotting. Constructs vvE3L-Rev, SPPV034/D1-G801S, and SPPV034/D12-S131N express intermediate and late proteins (Figure 3.2A(ii)), while the translation of the intermediate and late proteins was severely blocked in vvΔE3L, vvΔE3/SPPV034, vvΔE3/D1-G801S and vvΔE3/D12-S131N. Additionally, relative protein abundance correlates well with virus yield, particularly using a low MOI infection (Figure 3.2A(ii) and B(i)). SPPV034/D1-G801S and SPPV034/D12-S131N express a lower level of intermediate and late proteins and produce lower virus yields compared to vvE3L-Rev. Early protein expression was comparable among all constructs at late stages of infections (8 hpi) however, vvΔE3/SPPV034 and vvΔE3/D12-S131N express less vvD12 protein at early stages of infection (5 hpi) (Figure 3.2A(ii)).

As PKR is one of the major antiviral response mechanisms of HeLa cells, the replication capacity of these viruses was also examined using HeLa PKR knockout cells (Figure 3.3A(i)). Viruses vvΔE3L and vvΔE3/SPPV034 did replicate in HeLa PKR knockout cells, albeit to lower levels than vvE3L-Rev, SPPV034/D1-G801S, and SPPV034/D12-S131N. Interestingly, constructs vvΔE3/D1-G801S and vvΔE3/D12-S131N replicated approximately 10-fold (1 log value) and 5-fold less than vvΔE3L, respectively. Early, intermediate and late viral gene expression was also examined in HeLa PKR knockout cells (Figure 3.3A(ii)).

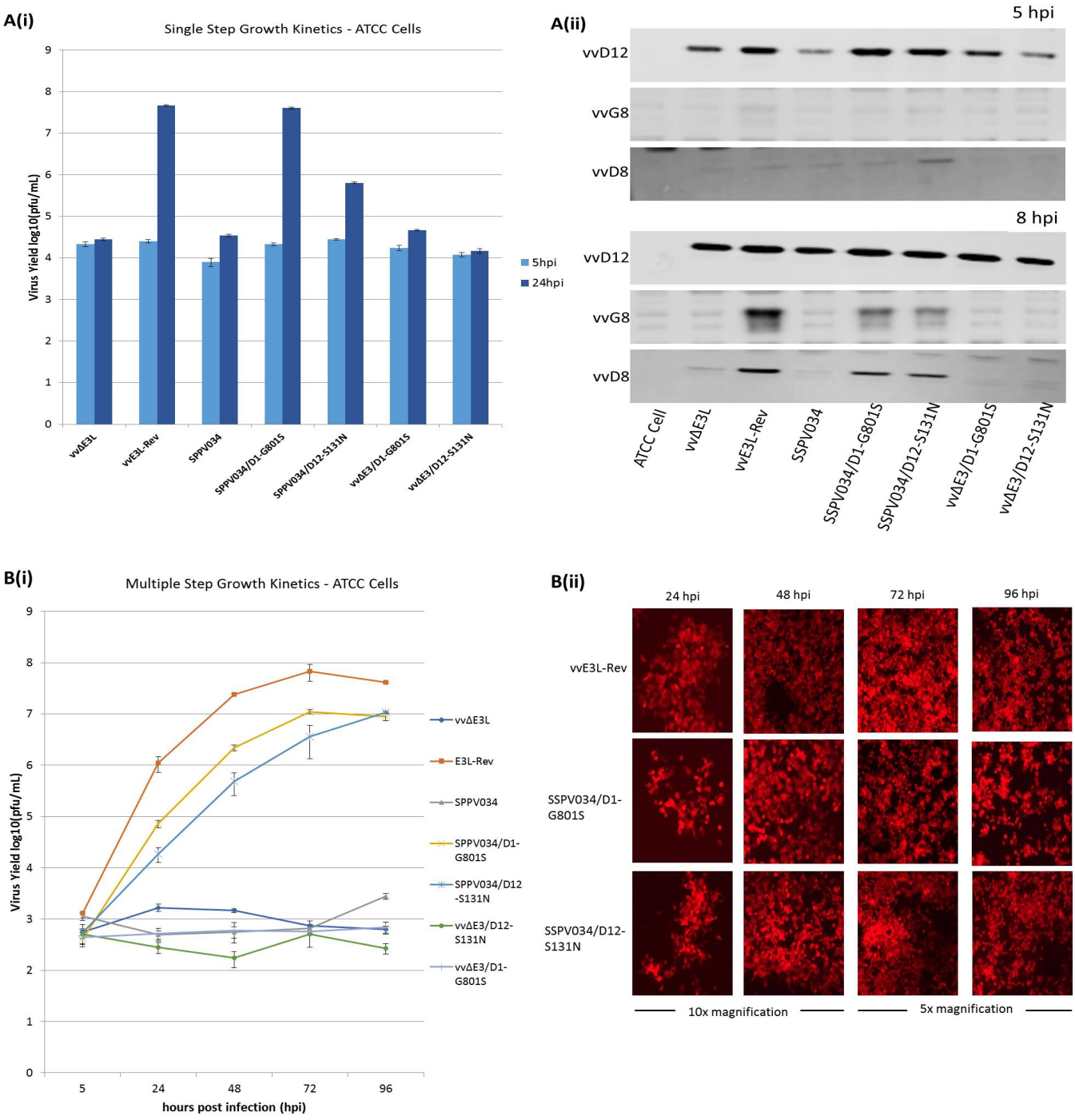


Figure 3.2 The D1-G801S and D12-S131N mutations rescues replication of the SSPV034 recombinant virus in HeLa cells. (**A**) Replication efficiency in a non-permissive cell line was determined using single step growth kinetics. HeLa cells were infected at MOI 5 and harvested at 5 and 24 hpi. Virus yield was determined using a plaque assay in BHK21 cells (**i**). Data is representative of two independent experiments and error bars represent the standard error of the mean. VACV gene expression was determined using western blotting against early (vvD12), intermediate (vvG8) and late (vvD8) VACV protein antibodies (**ii**). (**B**) Replication efficiency in non-permissive cells was also investigated using multi-step growth kinetics. HeLa cells were infected at MOI 0.1 and harvested at 5, 24, 48, 72 and 96 hpi. Virus yield was determined using a plaque assay in BHK21 cells (**i**). Data is representative of two independent experiments and error bars represent standard error of the mean. Pictures demonstrating plaque growth were obtained using UV microscopy at 24, 48, 72 and 96 hpi for vvE3L-Rev, SPPV034/D1-G801S and SPPV034/D12-S131N constructs (**ii**).

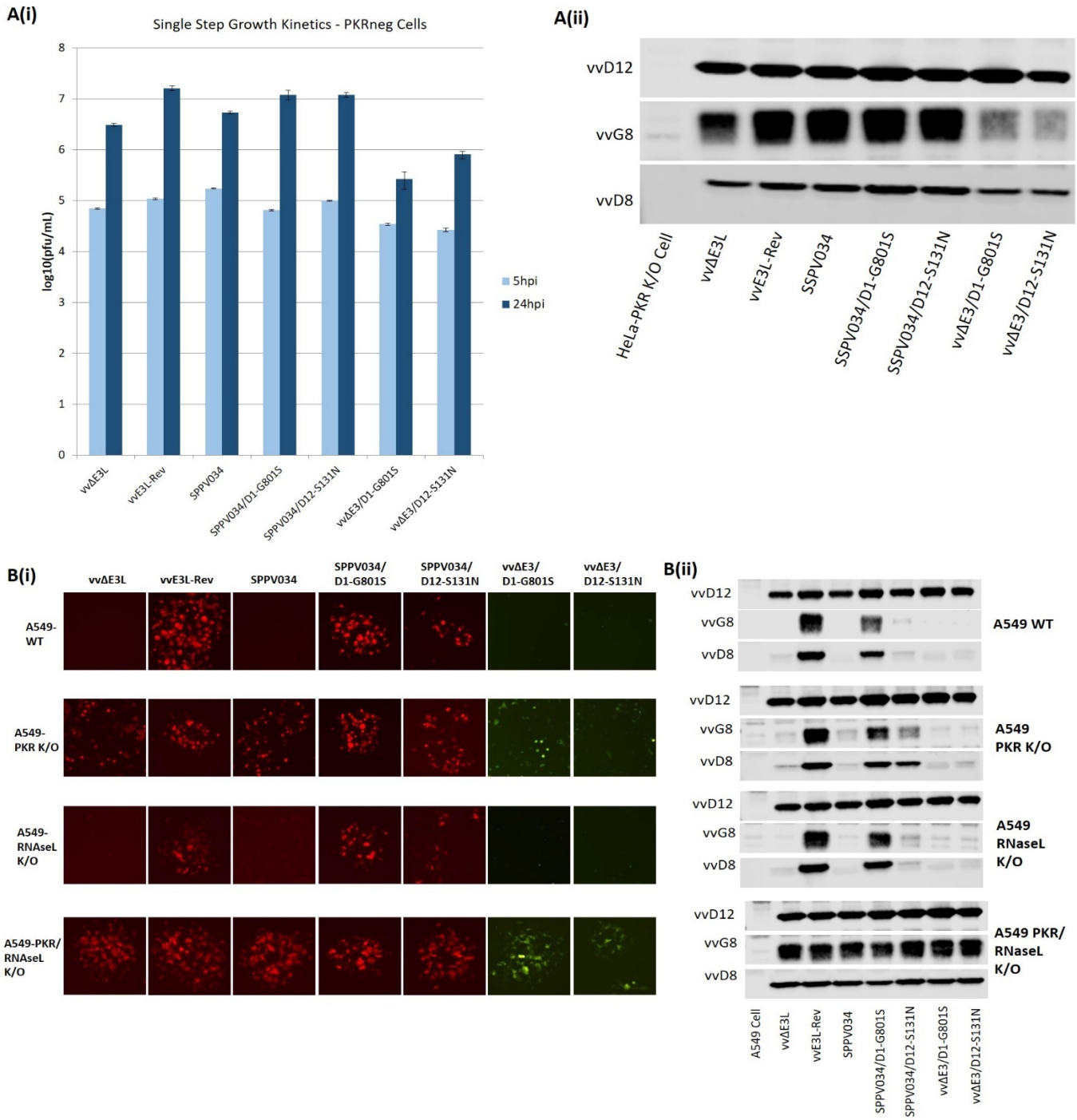


Figure 3.3. The D1-G801S mutation antagonizes both the PKR and RNAseL pathways to rescue replication of the SPPV034 recombinant virus, whereas the D12-S131N mutation antagonizes the RNAseL pathway only.
(**A**) Replication efficiency in a permissive cell line was determined using single step growth kinetics. HeLa PKR knockout cells were infected at MOI 5 and harvested at 5 and 24 hpi. Virus yield was determined using a plaque assay in BHK21 cells (**i**). Data is representative of two independent experiments and error bars represent the standard error of the mean. VACV gene expression was determined using western blotting against early (vvD12), intermediate (vvG8) and late (vvD8) vaccinia virus protein antibodies (**ii**). (**B**) Effects of PKR and RNAseL pathways on virus replication was analyzed using A549 cells. Cells were infected at MOI 1 and plaque formation was analyzed using UV microscopy (**i**). VACV gene expression was determined using western blotting against early (vvD12), intermediate (vvG8) and late (vvD8) VACV protein antibodies (**ii**).

Due to low RNAseL activity, PKR is the primary antiviral sensor for HeLa cell lines. To determine the role of the capping enzyme mutations in antagonizing other antiviral sensors alveolar basal epithelial (A549) cells and its derivatives were also used to measure virus replication efficacy. Wildtype A549 cells (A549-WT) and A549 cells lacking either PKR (A549-PKR K/O), RNAseL (A549-RNAseL K/O) or both (A549-PKR/RNAseL K/O) were infected using serial dilutions and plaque formation was monitored under UV microscopy (Figure 3.3B(i)). Plaques could be observed at 24 hpi. Viruses vvE3L-Rev and SPPV034/D1-G801S demonstrated large plaque formation in all A549 cell lines. Interestingly, SPPV034/D12-S131N demonstrated large plaque formation in A549-PKR/RNAseL K/O cells, intermediate sized plaques in A549-PKR K/O cells and restricted plaque formation in A549-WT and A549-RNAseL K/O cells. This indicates that the mutation in D12L demonstrates a greater ability for antagonizing RNAseL activity than PKR activity in A549 cells. Viruses vvΔE3L and vvΔE3/SPPV034 exhibited large plaque formation in A549-PKR/RNAseL K/O cells, limited plaque formation in A549-PKR K/O cells and no plaque formation in A549-WT and A549-RNAseL K/O cells. Viruses vvΔE3/D1-G801S and vvΔE3/D12-S131N exhibited plaque formation only in A549-PKR/RNAseL K/O cells. This indicates that the interaction between E3 family proteins and its cofactor, the mRNA capping enzyme, may be required for full antagonism of both the RNAseL and PKR mediated antiviral response pathway.

Early, intermediate and late vaccinia virus gene expression was used to confirm virus replication capabilities in A549 cells. Cells were infected, and protein expression was measured at 8 hpi using western blotting. Early protein expression was detected from all viruses in all A549 cell lines (Figure 3.3B(ii)). Viruses vvE3L-Rev and SPPV034/D1-G801S displayed intermediate and late protein expression in all A549 cell lines. SPPV034/D12-S131N demonstrated considerable intermediate and late protein expression in A549-PKR K/O and A549-PKR/RNAseL K/O cells, while small amounts of these proteins could be detected in A549-WT and A549-RNAseL K/O cells. Ample intermediate and late protein expression could only be detected in A549-PKR/RNAseL K/O cell infections for viruses vvΔE3L, vvΔE3/SPPV034, vvΔE3/D1-G801S and vvΔE3/D12-S131N. Slight protein expression could be detected in A549-PKR K/O cell infections for vvΔE3L and vvΔE3/SPPV034 viruses.

3 The SPPV034/D1-G801S construct inhibits activation of PKR and eIF2-α, while the SPPV/D1-G801S and SPPV034/D12-S131N constructs inhibit PARP and caspase 7 activation

The inhibition of PKR phosphorylation by E3 protein has been well established[94]. Here the phosphorylation of PKR and its downstream effector eIF2α was investigated using western blotting. HeLa cells were infected at an MOI of 5, and the total protein was collected at 5 and 8 hpi. The antibodies used to detect PKR demonstrate preferential binding for their respective forms (phosphorylated or unphosphorylated). However, both antibodies remain capable of detecting the other version, resulting in the double band pattern observed in Figure 3.4A. In comparison to vvΔE3/SPPV034, the SPPV034/D1-G801S construct was able to inhibit PKR activation and downstream phosphorylation of eIF2α. However, the vvΔE3/D1-G801S construct was not able to inhibit phosphorylation of PKR or eIF2α. These data indicate that the combination of the mutated D1R gene and SPPV034 is necessary to rescue the wild-type phenotype. An interesting trend is observed when comparing constructs with the wild-type mRNA capping enzyme versus constructs containing a mutation. SPPV034, which has the wild-type capping enzyme, demonstrates a large degree of PKR phosphorylation, whereas the mutation in D12L (S131N) slightly reduces the level of phosphorylation and the mutation in D1R (G801S) substantially reduces the level of phosphorylation. Likewise, vvΔE3L, which also has the wild-type capping enzyme, demonstrates a high level of PKR phosphorylation, whereas vvΔE3/D12-S131N demonstrates a slight reduction and vvΔE3/D1-G801S demonstrates a large reduction in phosphorylation.

Alternatively, cytosine arabinoside (ara-C) was used to inhibit virus genome replication, and activation of PKR was monitored. Ara-C acts as an antiviral agent by interfering with DNA synthesis via conversion to cytosine arabinoside triphosphate and inhibiting DNA and RNA polymerases[202]. Ara-C inhibits vaccinia virus genome replication, thereby preventing intermediate and late protein expression. HeLa cells were treated with or without ara-C and infected at MOI 5. Phosphorylation of PKR was measured at 3 and 10 hpi using western blotting (Figure 3.4B). Without ara-C treatment, virus infections behaved similarly to those carried out in Figure 3.4A. However, treatment with ara-C inhibited PKR activation for all viruses used

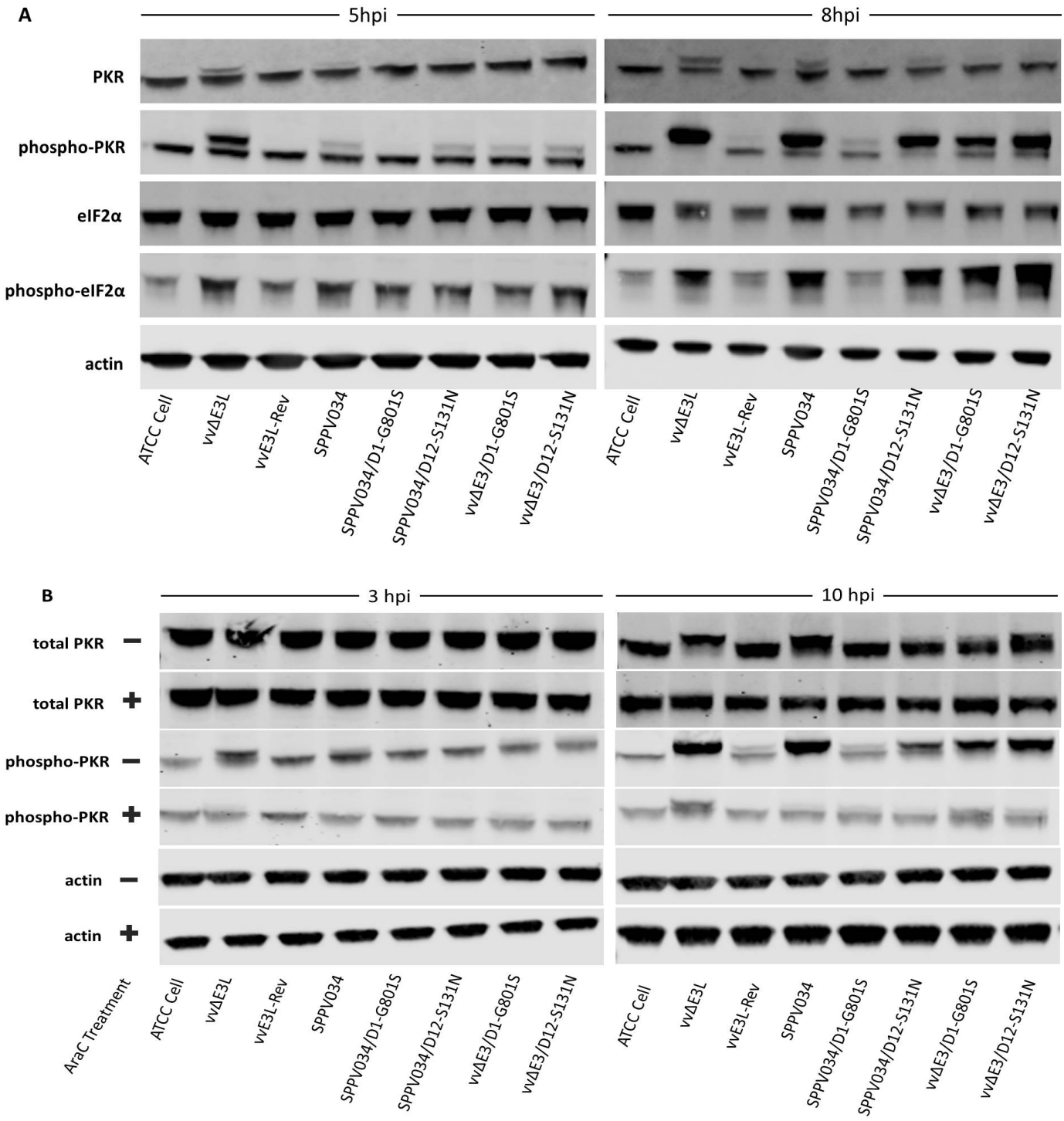


Figure 3.4. The SPPV034 recombinant virus with a D1-G801S mutation is able to inhibit PKR activation, while the SPPV034 recombinant virus with a D12-S131N mutation can not inhibit PKR activation.
(A) HeLa cells were infected at MOI 5 and protein was harvested at 5 and 8 hpi. The activation of antiviral pathways was measured using phosphorylation of PKR and eIF2α by western blotting. (B) HeLa cells were treated with or without cytosine arabinoside (ara-C), to inhibit virus genome replication, and infected at MOI 5. Protein was harvested at 5 and 8 hpi and PKR activation was measured using western blotting.

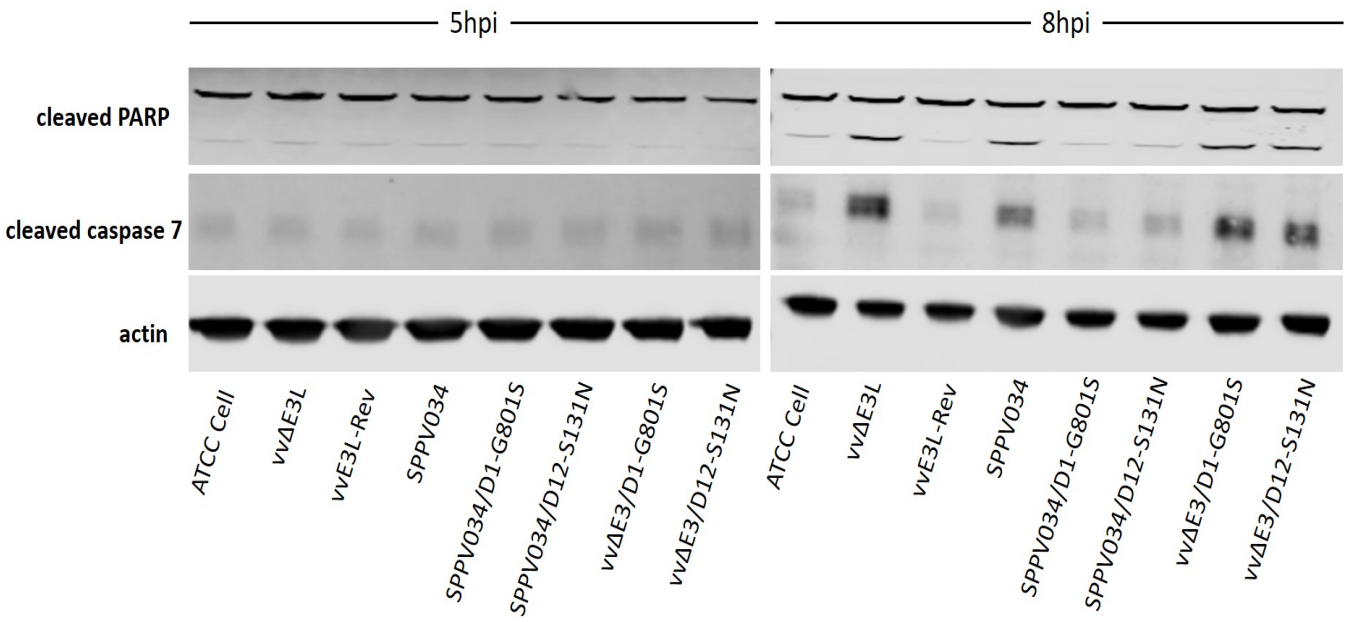


Figure 3.5. The SPPV034 recombinant virus with the D1-G801S mutation or the D12-S131N mutation is able inhibit activation of apoptosis by preventing cleavage of PARP and caspase 7.
HeLa cells were infected at MOI 5 and protein was harvested at 5 and 8 hpi. Induction of apoptosis was measured using cleavage of PARP and caspase 7 by western blotting. Actin was used as a load control in all experiments.

(Figure 3.4B). These data indicate that vaccinia virus modulates host antiviral responses during intermediate and late stages of replication.

Activation of the antiviral response pathway triggered by PKR leads to activation of apoptotic pathways through cleavage of caspase 7 and poly (ADP-ribose) polymerase (PARP)[94,203]. Therefore, the levels of these enzymes were also analyzed using western blotting (Figure 3.5). Interestingly, vvΔE3L, vvΔE3/SPPV034, vvΔE3/D1-G801S and vvΔE3/D12-S131N induced cleaved caspase 7 and PARP, while SPPV034/D1-G801S and SPPV034/D12-S131N were able to prevent cleavage. These data indicate that the ortholog, SPPV034, and the mutated D1 or D12 worked in conjunction to prevent apoptosis during infection.

4 Mutations in the mRNA capping enzyme of a SPPV034 recombinant vaccinia virus suppress the expression of cytokines

Although the E3 ortholog, SPPV034, is unable to restore host range function, we have previously demonstrated that it could mediate a certain degree of inhibition of several cytokines[132] (*Varga and Cao; manuscript in preparation*) comparing with the vvΔE3L virus. Therefore, the modulation of cytokine expression induced by SPPV034/D1-G801S and SPPV034/D12-S131N was investigated.

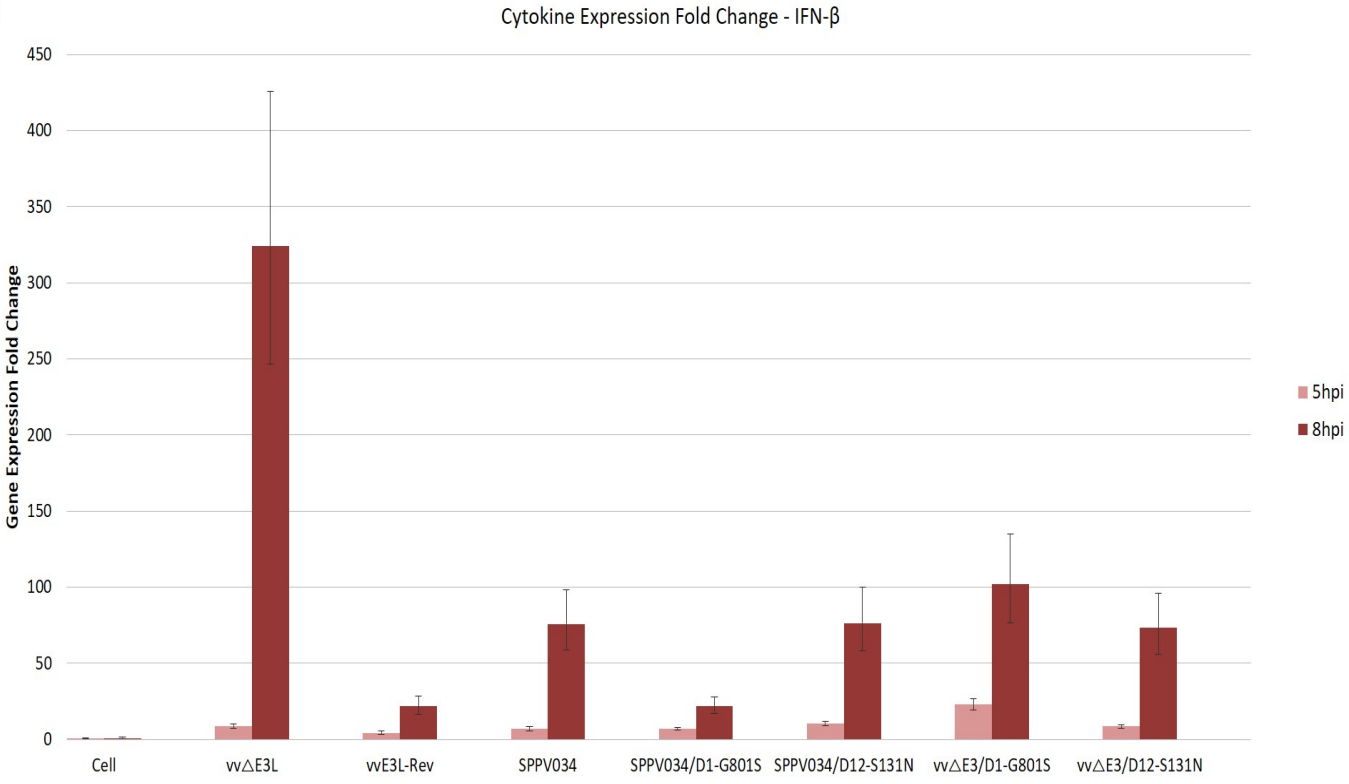
HeLa cells were infected at an MOI of 5, the total RNA was harvested at 5 and 8 hpi, and the mRNA of three representative cytokines, IFN-β, TNF-α, and IL-6, was quantified using RT-qPCR. As previously reported[132], vaccinia E3 mediated strong inhibition of expression of all three cytokines (vvE3L-Rev vs vvΔE3L) (Figure 3.6), while the sheeppox virus ortholog, SPPV034, inhibited cytokines to a lesser degree (SPPV034 vs vvΔE3L). All other constructs were able to down-regulate the cytokines, albeit to various degrees, compared to vvΔE3L. SPPV034/D1-G801S demonstrated stronger suppression of cytokines IFN-β, IL-6, and TNF-α, while the cytokine expression levels during SPPV034/D12-S131N infection was comparable to levels during SPPV034 infection (which has the wild-type D1 and D12 genes).

Interestingly, deletion of SPPV034 significantly compromised the ability of the vvΔE3/D1-G801S virus, to inhibit expression of the three cytokines, albeit vvΔE3/D1-G801S the inhibition was still slightly stronger than vvΔE3L (which contains the wild-type D1). On the other hand, deletion of SPPV034 did not significantly affect the expression of IFN-β or IL6, by

the virus vvΔE3/D12-S131N, while the TNF-α expression was reduced noticeably (vvΔE3/D12-S131N vs SPPV034/D12-S131N).

PKR plays a vital role in regulating expression of cytokines induced by virus infection, especially type I IFNs[204,205]. Therefore, the modulation of cytokines in HeLa PKR knockout cells was also investigated (Figure 3.7). HeLa PKR knockout cells were infected at an MOI of 5, RNA was harvested at 8 hpi and expression of IFN-β, TNF-α, and IL-6 was analyzed using RT-qPCR. IFN-β expression remained relatively unchanged in comparison to the infection in HeLa cells (Figure 3.6A vs Figure 3.7A). A noticeably lower level of TNF-α and IL-6 transcription was observed in the HeLa PKR knockout cells than in HeLa cells (Figure 3.6B and C vs Figure 3.7B and C). Interestingly, SPPV034 and vvΔE3/D1-G801S displayed greater increases of TNF-α expression compared to vvΔE3L in HeLa PKR knockout cells, with a 7-fold and 5-fold increase, respectively. The most notable increases to expression occurred for IL-6. SPPV034/D1-G801S and vvΔE3/D1-G801S suppressed expression to similar levels as vvE3L-Rev. vvΔE3/SSPV034, SSPV034/D12-S131N and vvΔE3/D12-S131N demonstrated partial suppression of IL-6 expression, reducing expression approximately 10-fold compared to vvΔE3L.

A



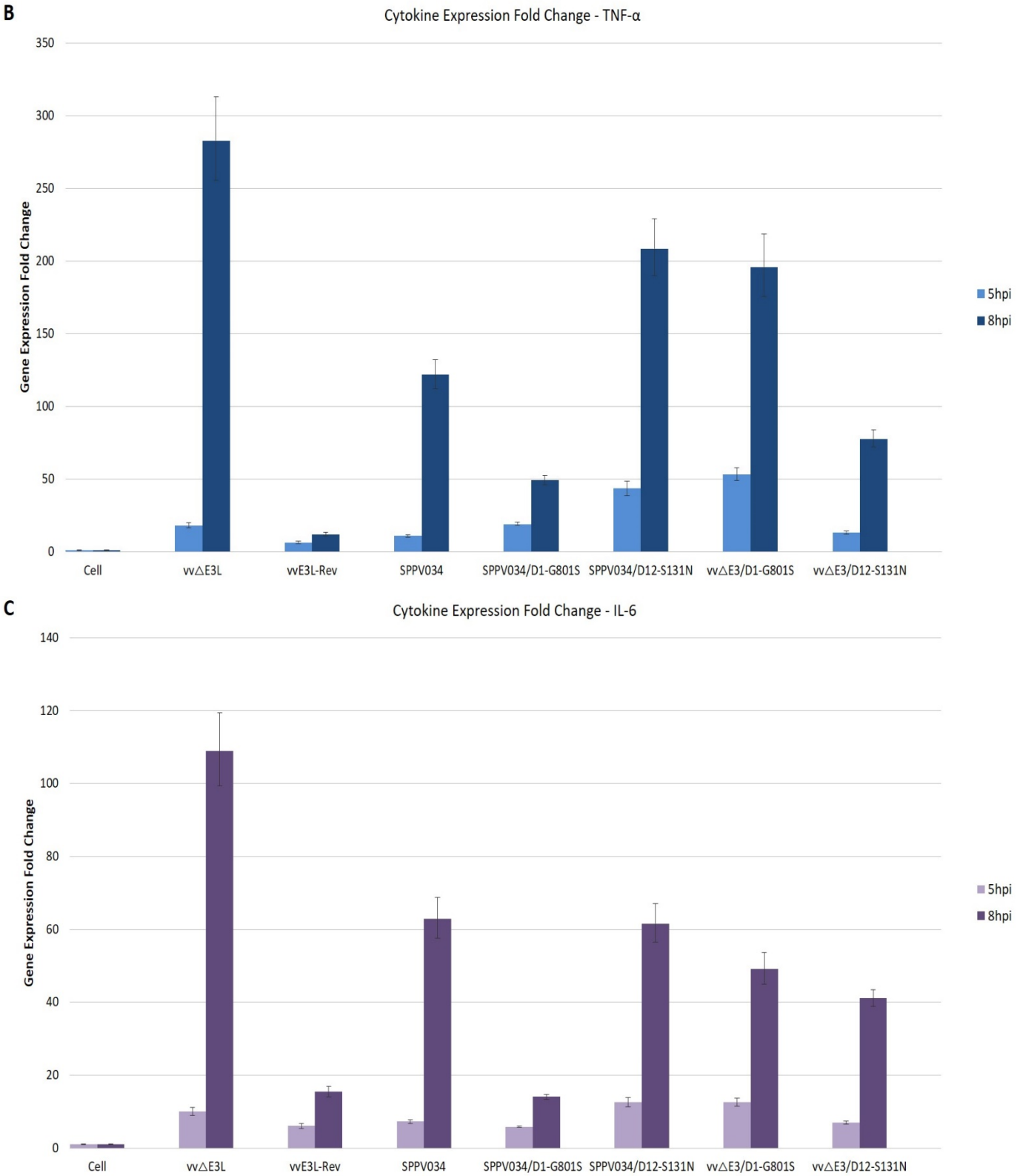
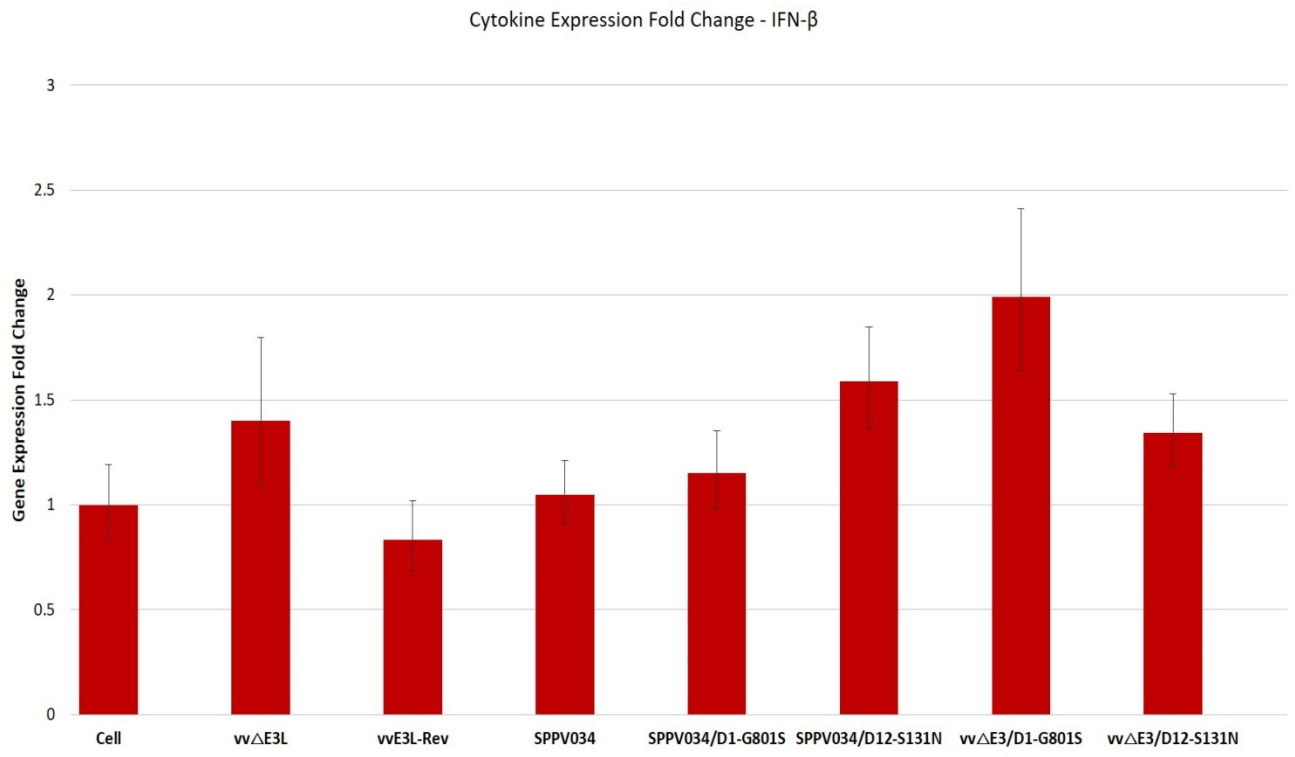
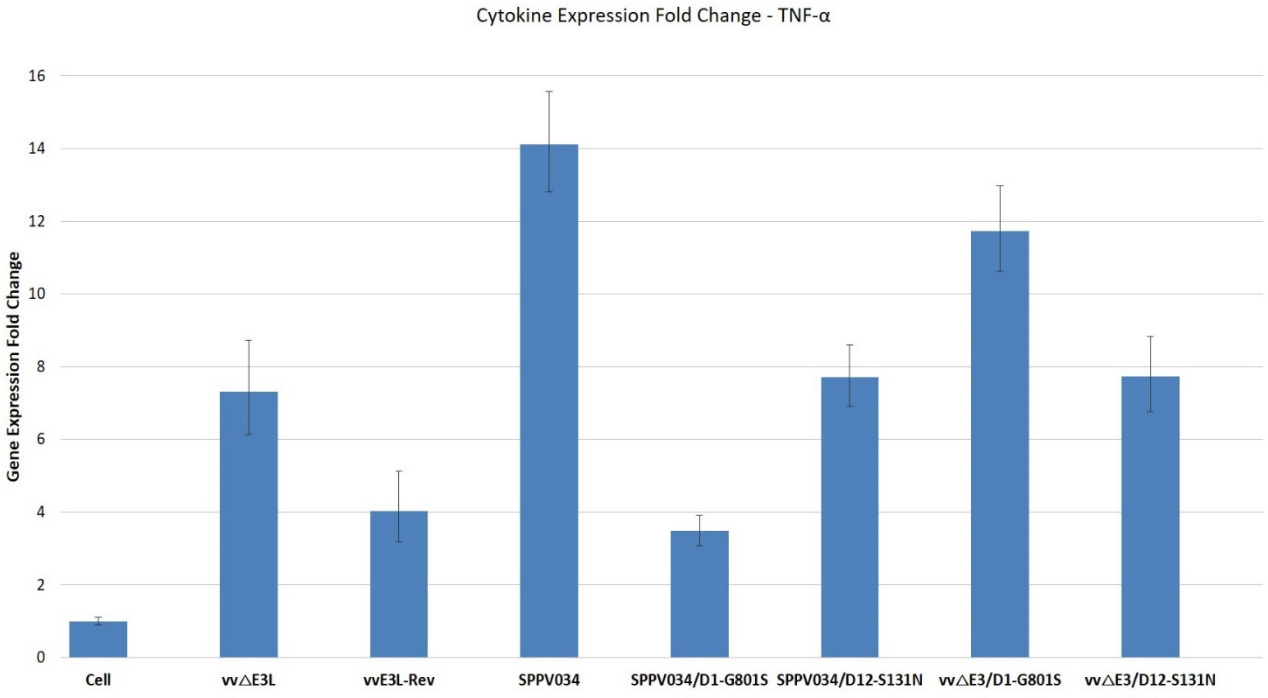


Figure 3.6. The D1-G801S mutation and D12-S131N mutation suppressed the activation of cytokines.
HeLa cells were infected at MOI 5 and RNA was collected at 5 and 8 hpi. Expression of cytokines IFN-beta (A), TNF-alpha (B), and IL-6 (C) were measured using RT-qPCR. Expression was normalized to actin and compared to uninfected cells. Error bars represent the standard error of the mean. Data is representative of two independent experiments.

A


Cytokine Expression Fold Change - IFN-β
Gene Expression Fold Change
3
2.5
2
1.5
1
0.5
0
Cell
vv△E3L
vvE3L-Rev
SPPV034
SPPV034/D1-G801S
SPPV034/D12-S131N
vv△E3/D1-G801S
vv△E3/D12-S131N

B


Cytokine Expression Fold Change - TNF-α
Gene Expression Fold Change
16
14
12
10
8
6
4
2
0
Cell
vv△E3L
vvE3L-Rev
SPPV034
SPPV034/D1-G801S
SPPV034/D12-S131N
vv△E3/D1-G801S
vv△E3/D12-S131N

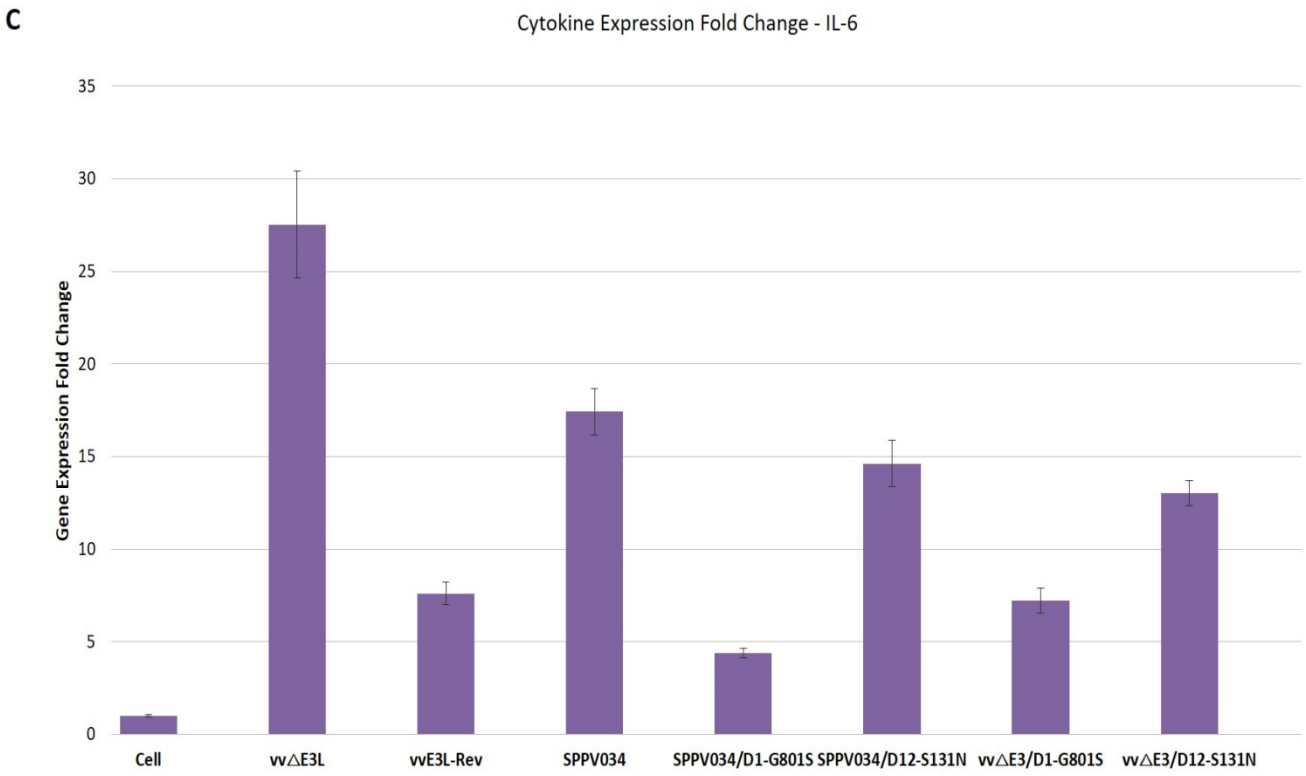


Figure 3.7. PKR is an important regulator of antiviral interferon responses during VACV infection in HeLa cells. HeLa PKR knockout cells were infected at MOI 5 and RNA was collected at 8 hpi. Expression of cytokines IFN-beta (A), TNF-alpha (B), and IL-6 (C) were measured using RT-qPCR. Expression was normalized to actin. Data is representative of two independent experiments and error bars represent standard error of the mean.

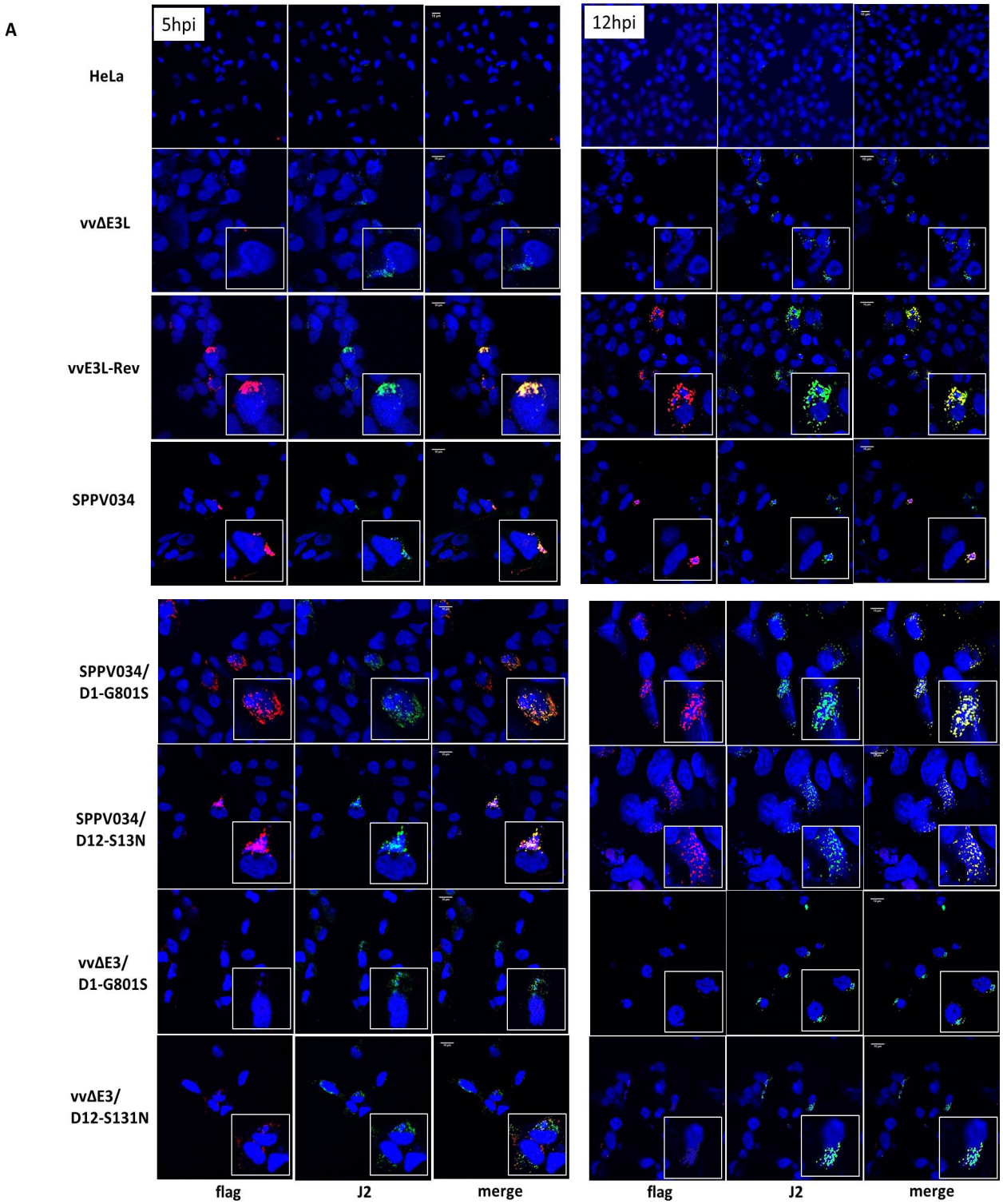
5 Mutations in the mRNA capping enzyme alter dsRNA production, but do not alter dsRNA localization within infected cells

dsRNA produced during a viral infection is a significant pathogen-associated molecular pattern (PAMP) responsible for inducing antiviral innate immune responses, such as IFN. In vaccinia virus infections, dsRNA is a by-product of intermediate and late stage replication[41]. dsRNA production and localization were investigated using a previously established antibody against dsRNA.

HeLa cells were infected at an MOI of 5, and fixed and stained at 5 and 24 hpi. Cells were imaged using confocal microscopy at 63X magnification under oil immersion. The cell nucleus and viral DNA were stained using DAPI (blue), a dsRNA-specific monoclonal antibody, J2, was used to detect dsRNA (green) and a flag antibody was used to detect vaccinia E3 and sheeppox virus SPPV034 (red) (Figure 3.8A). Both dsRNA and the proteins (E3 and SPPV034) could be detected at 5 hpi and expression increased over time only in the viruses capable of

replicating in HeLa cells (vvE3L-Rev, SPPV034/D1-G801S and SPPV034/D12-S131N). Both the E3 and SPPV034 co-localized well with dsRNA and the mutation in the D1 or D12 did not affect this co-localization.

PKR is the primary effector in blocking the intermediate and late stage protein translation of vvΔE3L and vvΔE3/SPV034[94,132] and this aborted replication results in significantly reduced late viral RNA synthesis in HeLa cells. Therefore, localization of the viral dsRNA was further examined and compared using HeLa PKR knockout cells. From HeLa PKR knockout cell infections, comparable amounts of dsRNA would be produced from these viruses. HeLa PKR knockout cells were infected and processed as described above. As shown in Figure 3.8B, expression of dsRNA and dsRNA binding protein increase over time for all constructs except SPPV034, which remained similar from 5 to 12 hours. Additionally, the mutation in the D1 (G801S) and D12 (S131N) did not cause altered localization of the viral dsRNA, E3 and SPPV034 proteins (Figure 3.8B).


A
5hpi
12hpi
HeLa
vvΔE3L
vvE3L-Rev
SPPV034
SPPV034/
D1-G801S
SPPV034/
D12-S13N
vvΔE3/
D1-G801S
vvΔE3/
D12-S131N
flag
J2
merge
flag
J2
merge

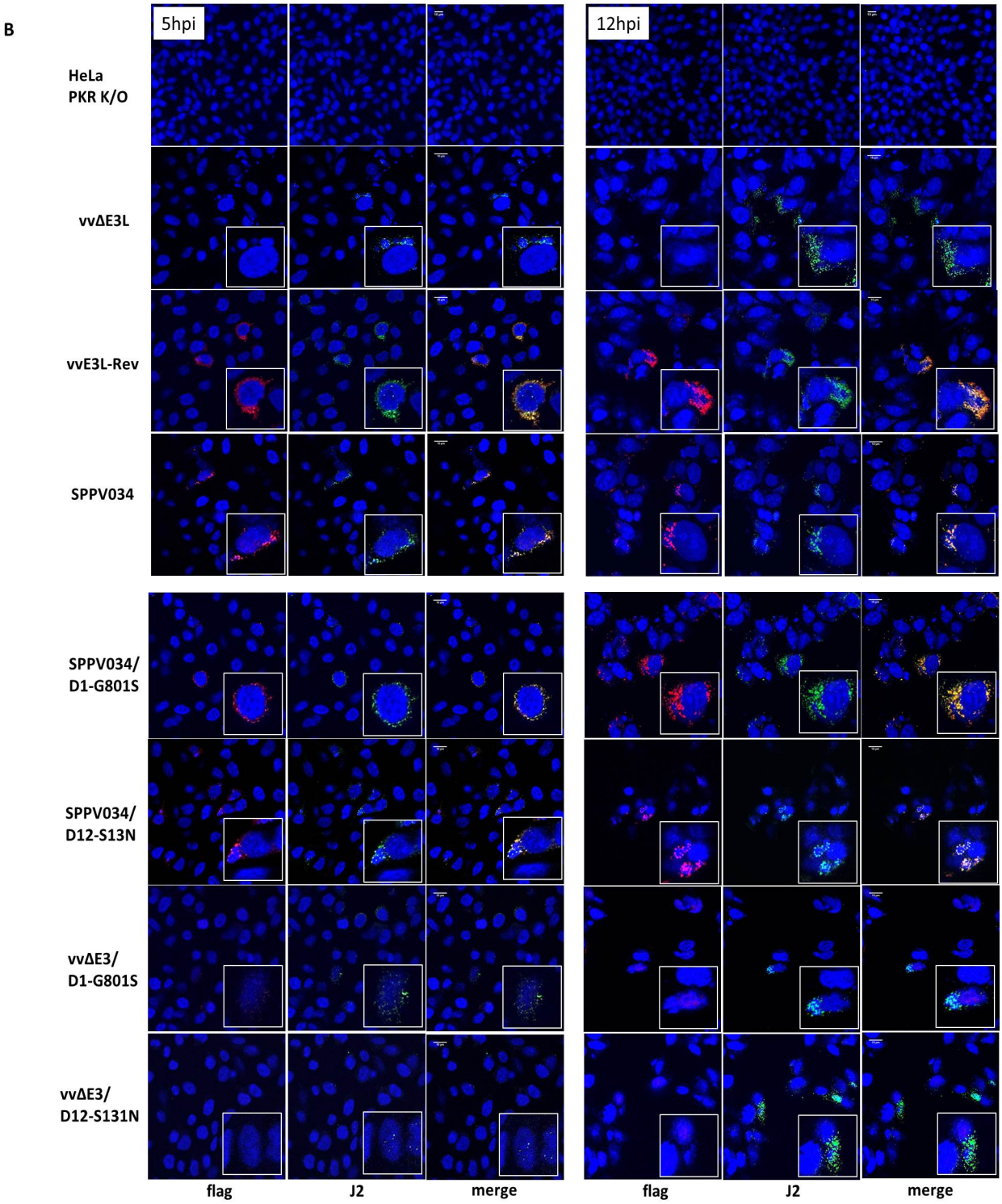


Figure 3.8. Mutations of the capping enzyme do not alter localization of dsRNA and E3 or SPPV034 proteins. Non-permissive HeLa cells (**A**) or permissive HeLa PKR knockout cells (**B**) were infected at MOI 5 and fixed with 3% paraformaldehyde at 5 and 12 hpi. Cells were permeabilized using 0.2% Triton X-100 and blocked using a 2% FBS, 2% BSA blocking buffer. A J2 antibody (SCICONS) was used to detect dsRNA and a flag antibody (Sigma) was used to detect the E3 protein or its ortholog SPPV034. Cells were imaged using a ZEISS confocal microscope at 63x magnification under oil immersion.

6 The differences in dsRNA production are quantitative and correlate with host cell species

It has been previously established that mutations in the decapping enzyme of vaccinia virus result in the accumulation of dsRNA and activation of antiviral responses[206]. In addition to their mRNA capping enzyme activities, vaccinia D1 and D12 are also essential for viral early transcription termination[186]. To assess whether mutations in the capping enzyme of vaccinia virus altered the transcription termination activity, a dot-blot was used to quantify dsRNA using a dsRNA specific monoclonal antibody, J2. HeLa cells were infected at an MOI of 5, and total RNA was harvested at 12 hpi. By 12 hours post infection, however, only the constructs capable of replicating in HeLa cells (vvE3L-Rev, SPPV034/D1-G801S and SPPV034/D12-S131N) demonstrated large increases in dsRNA production. The dsRNA level from SPPV034/D12-S131N infected HeLa cells was considerably lower than vvE3L-Rev and SPPV034/D1-G801S. Since most of viral dsRNA is produced at late stages of infection, after the viral DNA replication, HeLa cells were also treated with cytosine arabinoside (ara-C) to inhibit vaccinia genome replication and abolish transcription of intermediate and late genes. Thus, the potential effects of the mutated D1 and D12 on their early transcription termination activity can be examined in the presence of ara-C. Genome replication triggers the end of early gene transcription, therefore by blocking DNA replication with ara-C, early transcription could be extended so the low amount of dsRNA produced from early transcription could accumulate and be observed. Interestingly, when the intermediate and late transcription was blocked with ara-C, the dsRNA level only slightly varied among the different viruses, in that the SPPV034/D1-G801S produced slightly more dsRNA than the other viruses (Figure 3.9). Thus, it is unlikely that the mutation in the D1 or D12 altered their early transcription termination activity.

For the recombinant vaccinia viruses unable to replicate in HeLa cells, such as vvΔE3L and SPPV034, the late gene transcription is blocked. Thus, the impact of the mutation in D1 and D12 on the total dsRNA production cannot be examined. To this end, the total dsRNA level was also investigated in HeLa PKR knockout and BHK21 cells, in which all the recombinant viruses replicate in the same manner, described above. In HeLa PKR knockout cells, SPPV034 demonstrated the highest level of dsRNA (Figure 3.10). Constructs vvE3L-Rev, SPPV034/D1-G801S and SPPV034/D12-S131N showed an intermediate level of dsRNA production, while vvΔE3L, vvΔE3/D1-G801S and vvΔE3/D12-S131N showed considerably less dsRNA compared to the recombinant viruses expressing E3 or SPPV034. These data imply that the mutations in the

mRNA capping enzyme influence dsRNA quantity, at least in HeLa PKR knockout cells. SPPV034/D12-S131N demonstrates a slight decrease in dsRNA amount, while SPPV034/D1-G801S demonstrates a larger decrease in dsRNA when compared to SPPV034 (which has the wild-type version of the mRNA capping enzyme). This same trend is observed if we compare vvΔE3/D12-S131N (slight decrease) and vvΔE3/D1-G801S (larger decrease) to vvΔE3L. These data also indicate that the D1 mutation (G801S) is more influential in decreasing the amount of dsRNA produced during infection in HeLa PKR knockout cells than the D12 mutation (S131N).

Infections in BHK21 cells also displayed altered dsRNA quantity compared to HeLa or HeLa PKR knockout infections (Figure 3.11). Infection with SPPV034/D12-S131N demonstrated the largest production of dsRNA in BHK21 cells, while vvΔE3/D1-G801S demonstrated the lowest quantity of dsRNA produced. Treatment of BHK21 cells with ara-C and blockade of intermediate and late transcription resulted in drastically decreased dsRNA production in all constructs. These results indicate that dsRNA quantity is cell line dependent as the mutations in the capping enzyme (D1-G801S or D12-S131N) did not result in any clear trends in the quantity of dsRNA.

7 Late RNA species generated from vaccinia virus infection induce antiviral responses when transfected into HeLa cells

Previously, we have shown that vaccinia virus dsRNA products could directly induce antiviral responses including PKR activation and IFN-β expression[132,207]. To this end, the antiviral responses induced by the RNA species generated from constructs containing a mutation in a mRNA capping enzyme subunit was investigated. HeLa PKR knockout cells were infected at MOI 5, and the total RNA was collected at 12 hpi. HeLa cells were transfected with 3 μg of total RNA. HeLa PKR knockout RNA was used as the control.

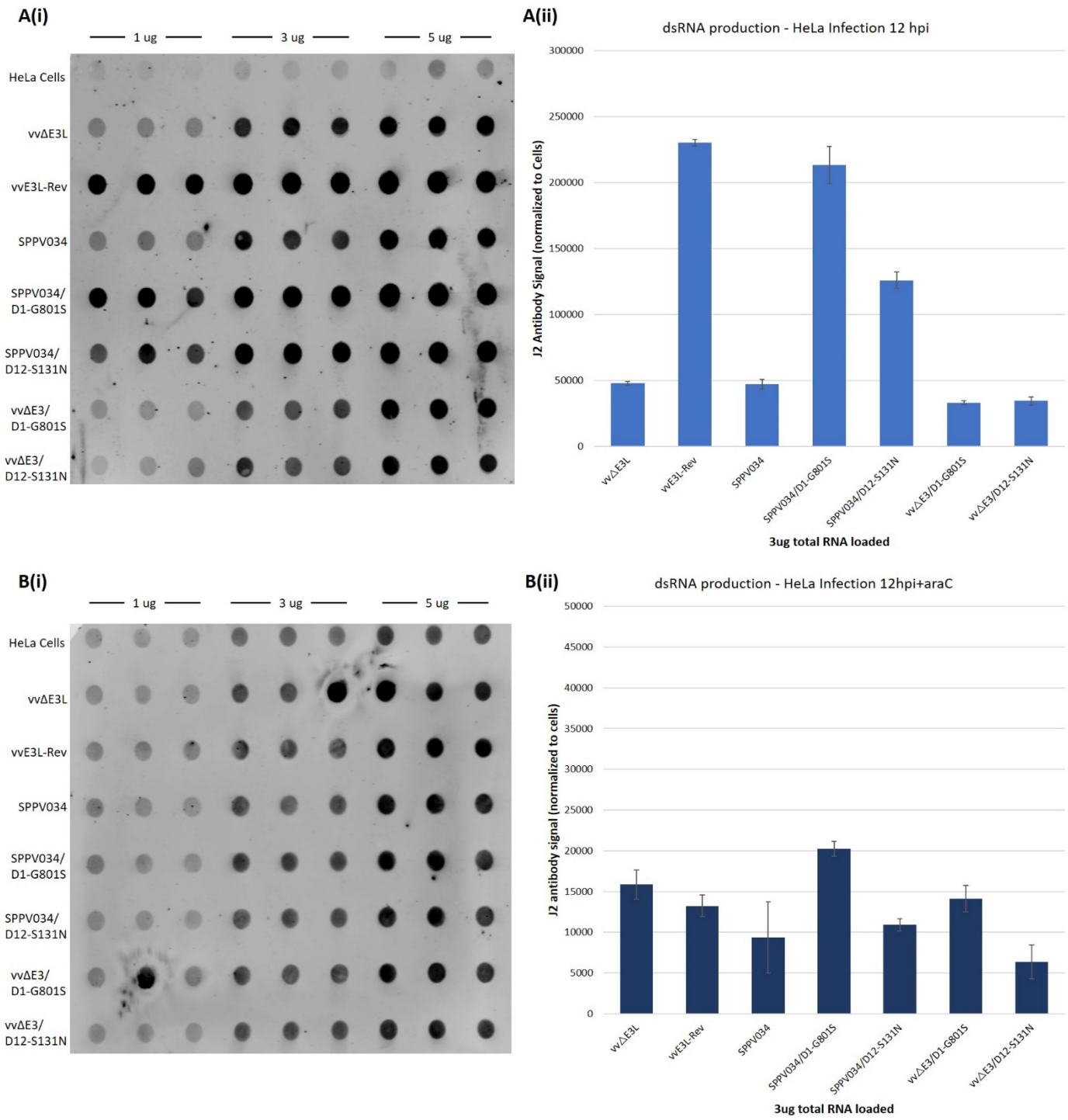


Figure 3.9. Increased levels of dsRNA are observed during infections with viruses capable of replicating in HeLa cells. HeLa cells were treated with or without ara-C to inhibit virus genome replication, infected at MOI 5 and RNA was collected at 12hpi. RNA was hybridized to a nitrocellulose membrane in 1, 3 and 5 µg amounts using a dot-blot apparatus (BioRad). Membranes were probed for dsRNA using a J2 antibody and the signal was analyzed using Image Studio software. Signal intensity was normalized to uninfected cells. Error bars represent the standard error of the mean. Data is representative of two independent experiments. A (i & ii) nitrocellulose membrane containing RNA and dsRNA amounts in 3µg of total RNA from HeLa cell infections at 12 hpi without ara-C treatment. B (i & ii) nitrocellulose membrane containing RNA and dsRNA amounts in 3µg of total RNA from HeLa cell infections at 12 hpi with ara-C treatment.

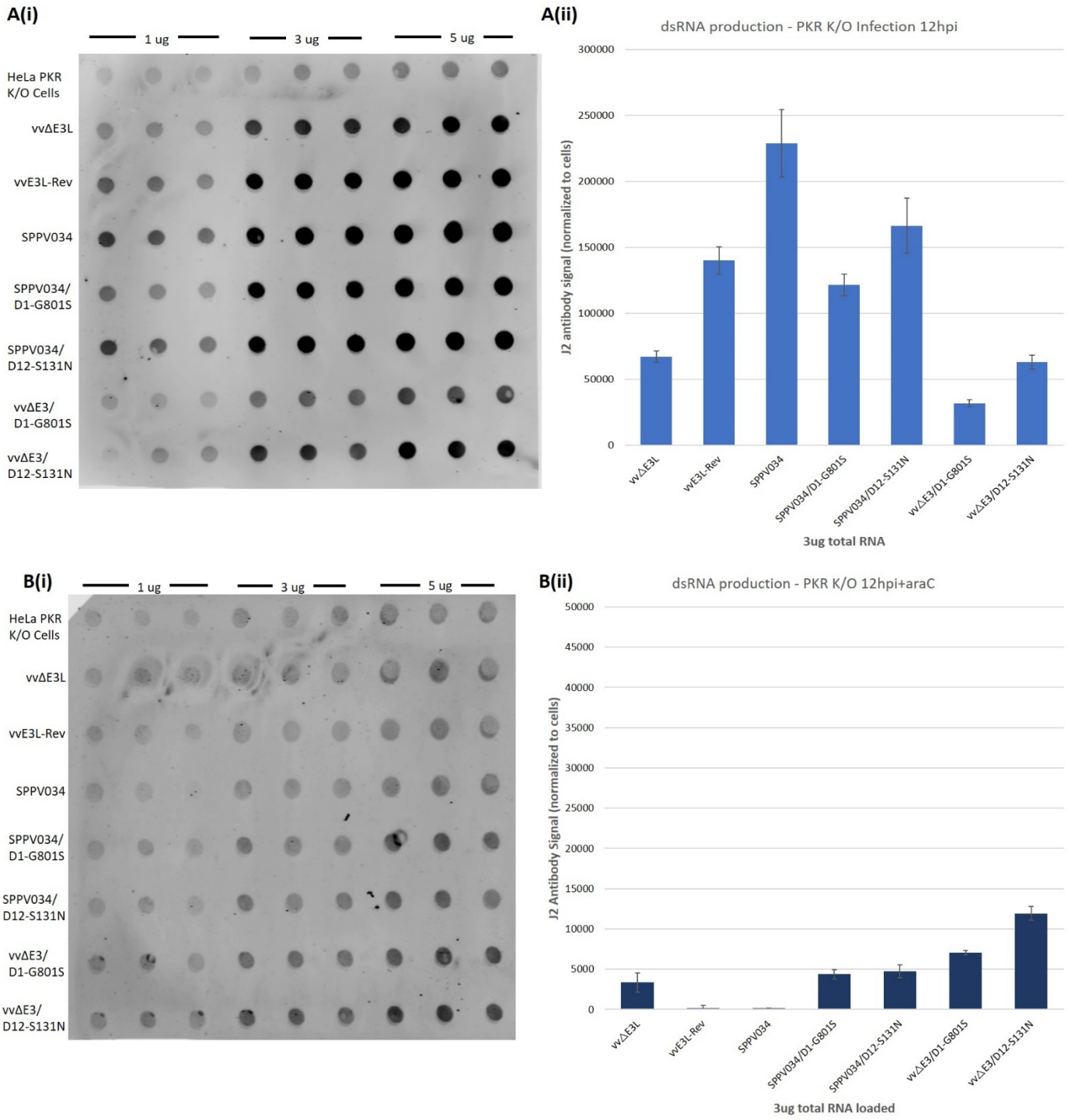


Figure 3.10. D1-D12 heterodimer mutations decrease levels of dsRNA produced during HeLa PKR knockout cell infections. HeLa PKR knockout cells were treated with or without ara-C to inhibit virus genome replication, infected at MOI 5 and RNA was collected at 12hpi. RNA was hybridized to a nitrocellulose membrane in 1, 3 and 5 µg amounts using a dot-blot apparatus (BioRad). Membranes were probed for dsRNA using a J2 antibody and the signal was analyzed using Image Studio software. Signal intensity was normalized to uninfected cells. Error bars represent the standard error of the mean. Data is representative of two independent experiments. A (i & ii) nitrocellulose membrane containing RNA and dsRNA amounts in 3µg of total RNA from HeLa PKR knockout cell infections at 12 hpi without ara-C treatment. B (i & ii) nitrocellulose membrane containing RNA and dsRNA amounts in 3µg of total RNA from HeLa PKR knockout cell infections at 12 hpi with ara-C treatment.

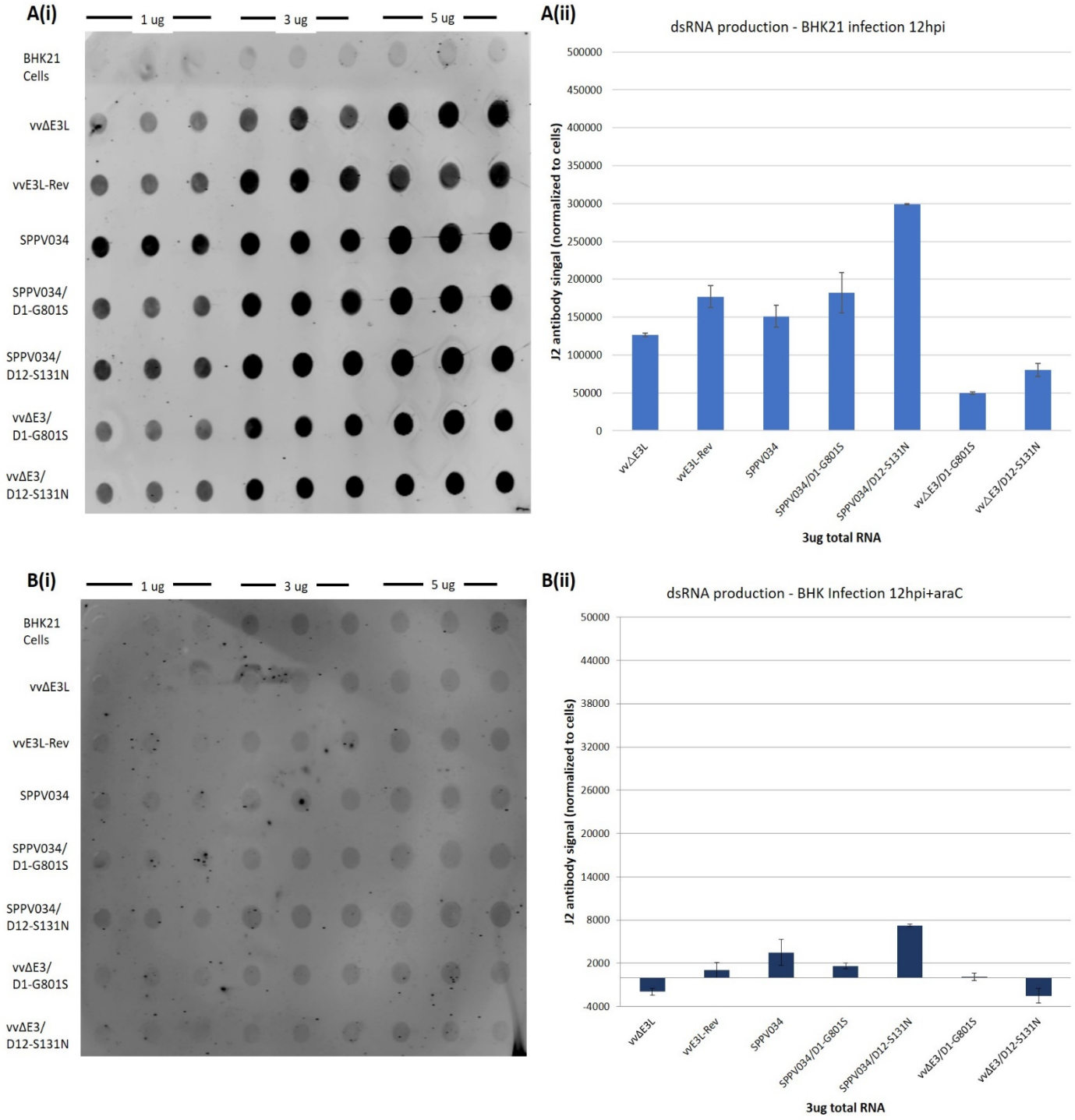


Figure 3.11. Viral dsRNA production is altered based on host cell line used for infection.
BHK21 ells were treated with or without ara-C to inhibit virus genome replication, infected at MOI 5 and RNA was collected at 12hpi. RNA was hybridized to a nitrocellulose membrane in 1, 3 and 5 µg amounts using a dot-blot apparatus (BioRad). Membranes were probed for dsRNA using a J2 antibody and the signal was analyzed using Image Studio software. Signal intensity was normalized to uninfected cells. Error bars represent the standard error of the mean. Data is representative of two independent experiments. A (i & ii) nitrocellulose membrane containing RNA and dsRNA amounts in 3µg of total RNA from permissive BHK21 cell infections at 12 hpi without ara-C treatment. B (i & ii) nitrocellulose membrane containing RNA and dsRNA amounts in 3µg of total RNA from permissive BHK21 cell infections at 12 hpi with ara-C treatment.

PKR activation at 8 hours post transfection (hpt) was determined using Western blotting (Figure 3.12A). HeLa PKR knockout RNA did not induce PKR activation, and only late RNA species were capable of inducing activation. RNA from all the constructs except vvΔE3/D1-G801S induced phosphorylation of PKR, albeit to varying degrees. RNA from SPPV034 induced the greatest level of PKR phosphorylation, while RNA from vvΔE3L, vvE3L-Rev and SPPV034/D12-S131N induced lower levels of PKR phosphorylation. RNA from SPPV034/D1-G801S and vvΔE3/D12-S131N only slightly induced PKR phosphorylation. RNA from all constructs demonstrated some level of eIF2-α phosphorylation. Interestingly, the same trend of PKR phosphorylation between constructs bearing a capping enzyme mutation compared to the wild-type version was also demonstrated here. SPPV034/D12-S131N demonstrated a slight decrease in PKR phosphorylation, while SPPV034/D1-G801S demonstrated a larger decrease in PKR phosphorylation when compared to SPPV034 (which contains the wild-type capping enzyme). Furthermore, vvΔE3/D12-S131N also demonstrated a slight decrease in PKR phosphorylation, while vvΔE3/D1-G801S demonstrated a larger decrease in phosphorylation compared to vvΔE3L (which also contains the wild-type capping enzyme).

Expression of cytokines IFN-β, TNF-α and IL-6 at 8 hpt with RNA extracted from HeLa PKR knockout cell infections were analyzed using RT-qPCR. HeLa PKR knockout RNA was capable of inducing increases in cytokine expression compared to untransfected cells (data not shown). Since this experiment is focusing on the differences induced by viral RNA species, gene expression was compared to transfections using the RNA from mock-infected cells as these samples will contain the same host RNA species capable of altering cytokine expression. RNA from all constructs except vvΔE3/D1-G801S demonstrated increases of all cytokines analyzed (Figure 3.13), although to varying degrees. The same trend mentioned above was also observed in these results. These data strongly indicate that the mutations in the mRNA capping enzyme influence the amount of dsRNA produced during virus infection.

Since host cell species appears to have a role in determining dsRNA quantity, PKR activation from transfection of RNA from BHK21 cells was also analyzed as described above (Figure 3.12B). BHK21 cells were infected at MOI 5, and total RNA was collected at 12 hpi.

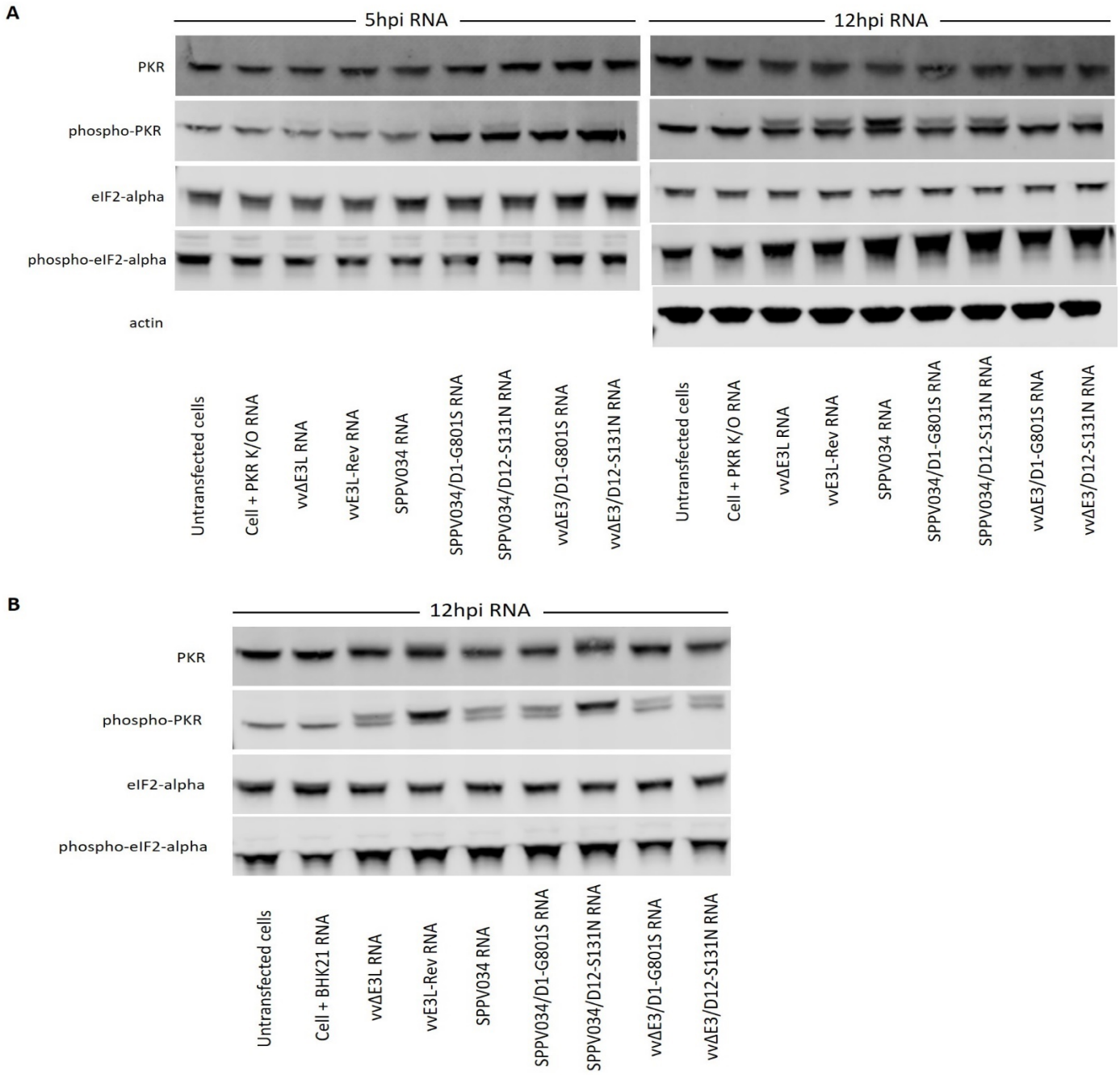
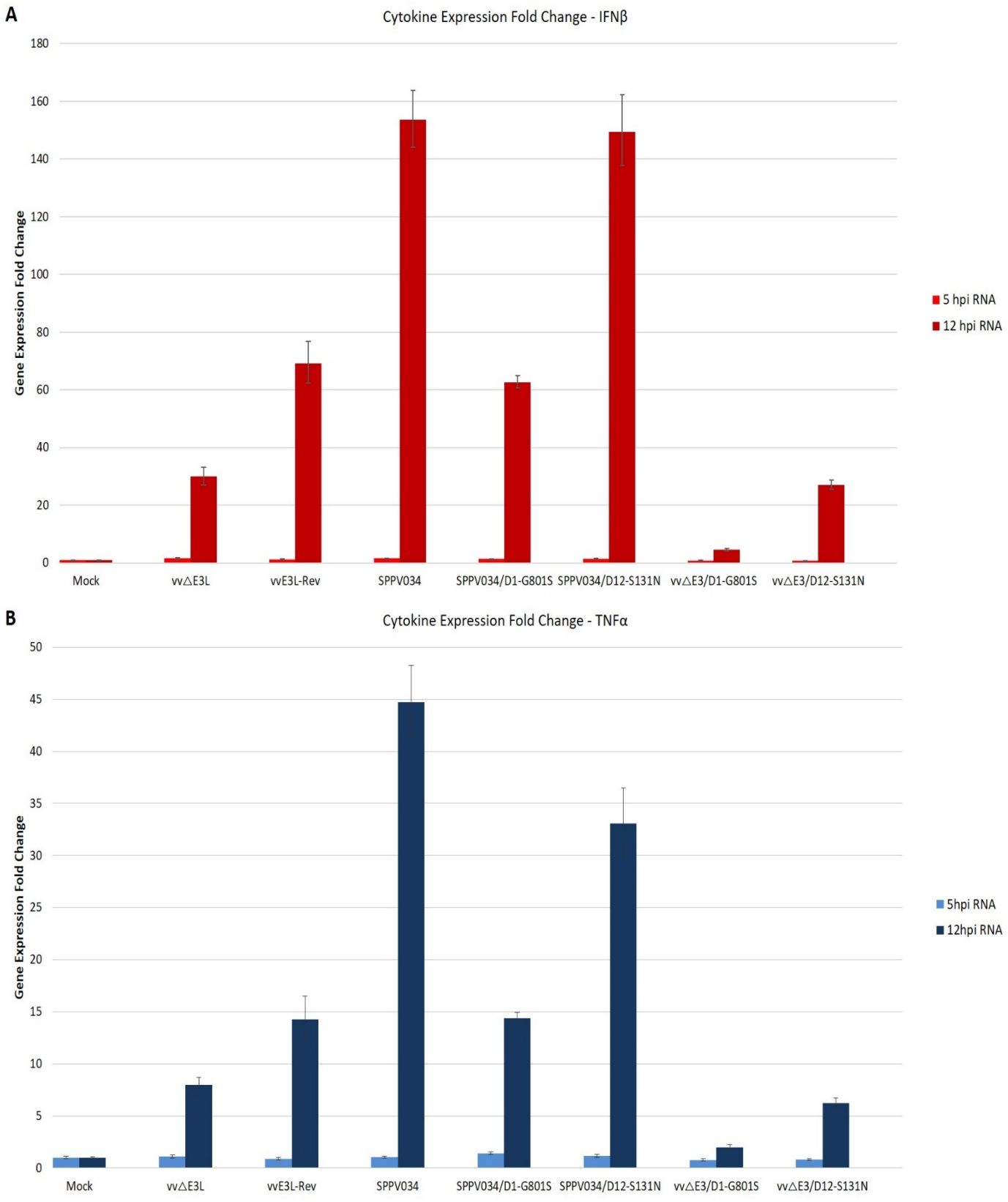


Figure 3.12. Late viral RNA products induce antiviral response pathways in host cells.
(**A**) HeLa PKR knockout cells were infected at MOI 5 and total RNA was harvested at 5 and 12 hpi. HeLa cells were transfected with 3µg of total RNA isolated from HeLa PKR knockout cell infections. Protein was harvested from HeLa transfections at 8 hours post transfection (hpt) and activation of antiviral responses was measured using phosphorylation of PKR and eIF2α by western blotting. (**B**) BHK21 cells were infected at MOI 5 and total RNA was harvested at 12hpi. HeLa cells were transfected with 3µg of total RNA isolated from BHK21 cells. Protein was harvested from HeLa transfections at 8 hpt and activation of antiviral responses was measured using phosphorylation of PKR and eIF2α by western blotting.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Gene Expression Fold Change
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160
140
120
100
80
60
40
20
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SPPV034
SPPV034/D1-G801S
SPPV034/D12-S131N
vvΔE3/D1-G801S
vvΔE3/D12-S131N
5 hpi RNA
12 hpi RNA
B
Cytokine Expression Fold Change - TNFα
Gene Expression Fold Change
50
45
40
35
30
25
20
15
10
5
0
Mock
vvΔE3L
vvE3L-Rev
SPPV034
SPPV034/D1-G801S
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vvΔE3/D12-S131N
5hpi RNA
12hpi RNA

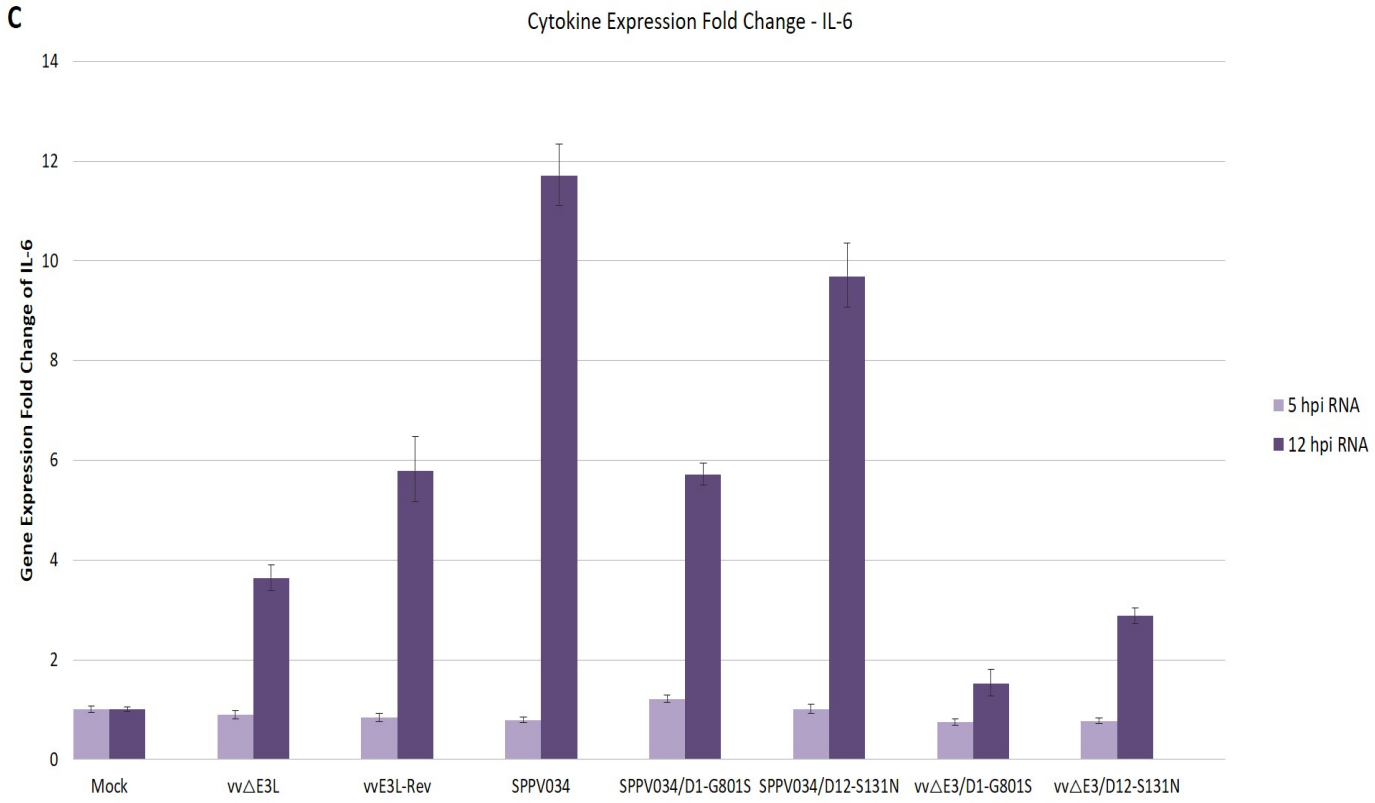
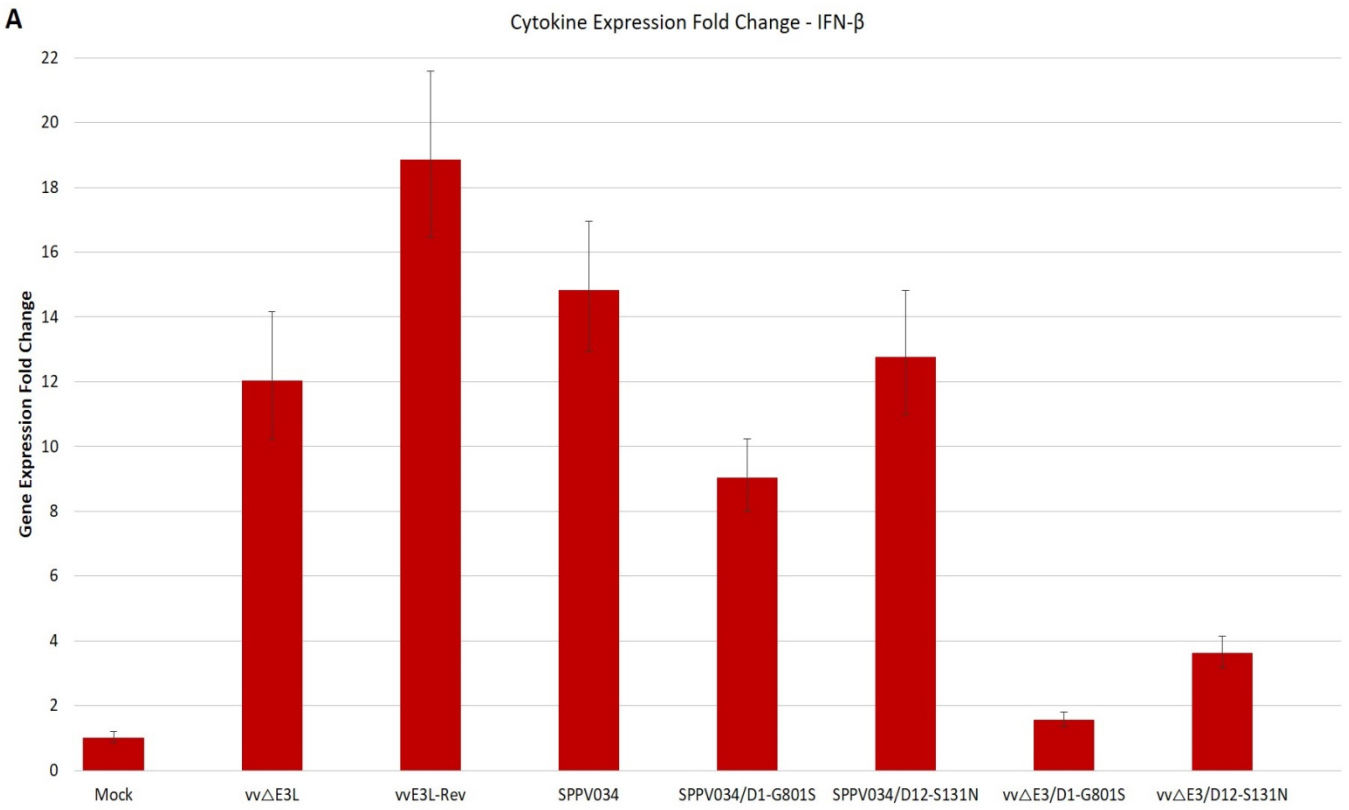


Figure 3.13. Late viral RNA products induce a variety of cytokines in host cells.
HeLa PKR knockout cells were infected at MOI 5 and total RNA was harvested at 5 and 12 hpi. HeLa cells were transfected with 3µg of total RNA isolated from HeLa PKR knockout cell infections. RNA was harvested from HeLa transfections at 8 hpt and cytokine expression was analyzed using RT-qPCR for cytokines IFNβ (A), TNF-α (B), and IL-6 (C). Gene expression was normalized to actin and compared to cells transfected with HeLa PKR knockout RNA only (mock). Data is representative of two independent experiments and error bars represent standard error of the mean.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Gene Expression Fold Change
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4
6
8
10
12
14
16
18
20
22
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vvE3L-Rev
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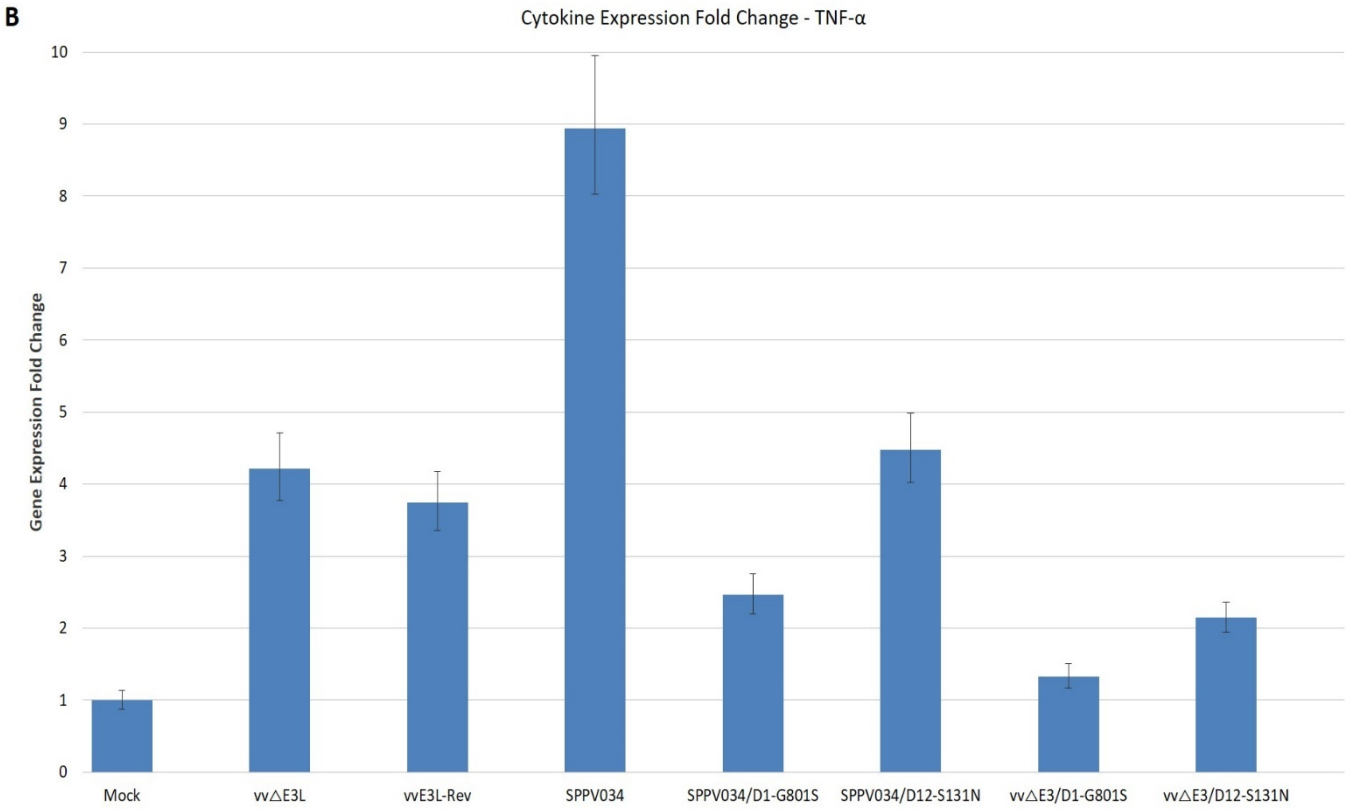
vv△E3/D12-S131N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Cytokine Expression Fold Change - TNF-α
Gene Expression Fold Change
0
1
2
3
4
5
6
7
8
9
10
Mock
vv△E3L
vvE3L-Rev
SPPV034
SPPV034/D1-G801S
SPPV034/D12-S131N
vv△E3/D1-G801S
vv△E3/D12-S131N

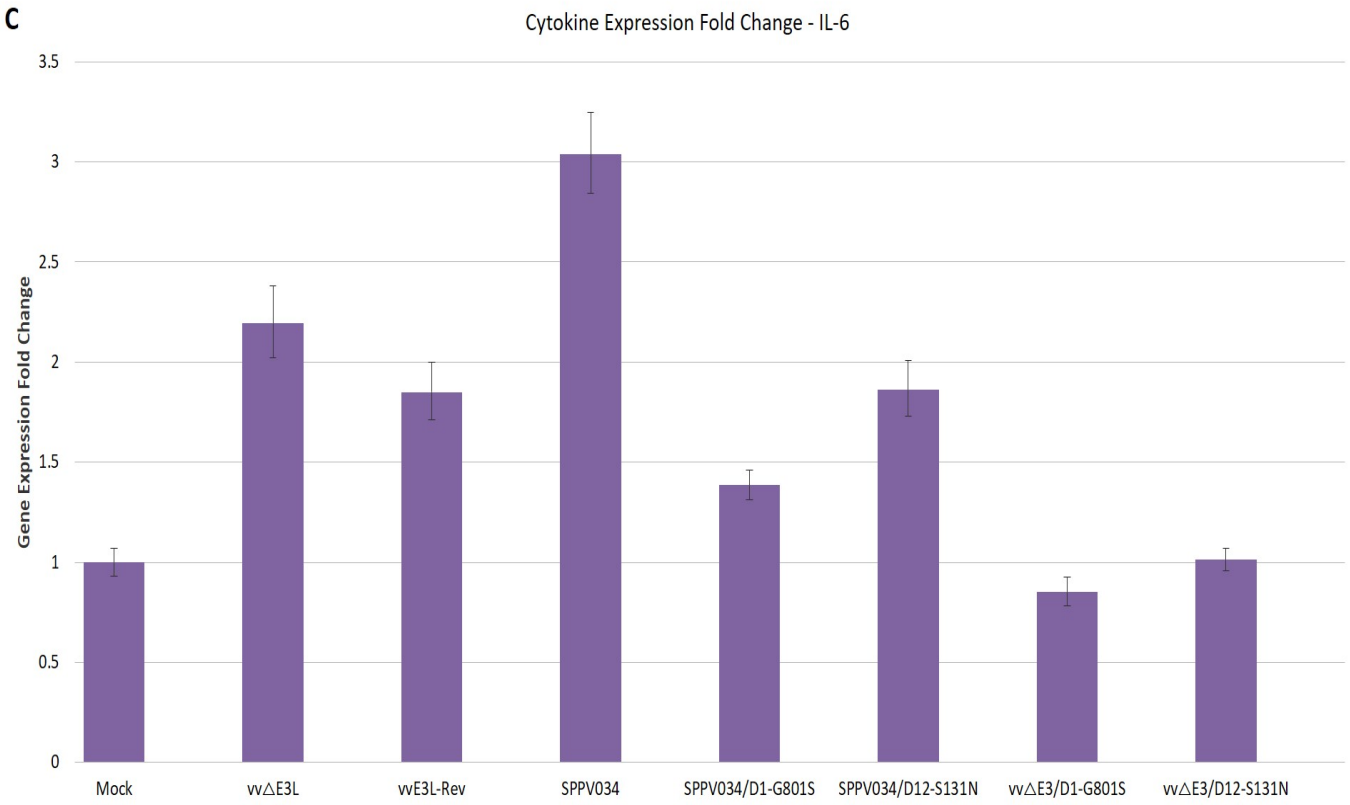

Figure 3.14. PKR is an important regulator of cytokine responses in HeLa cells.
HeLa PKR knockout cells were infected at MOI 5 and harvested at 12 hpi. PKR knockout cells were transfected with 3ug of RNA and RNA was harvested at 8 hpi. Gene expression of cytokines IFN-beta (A), TNF-alpha (B), and IL-6 (C) were measured by RT-qPCR. Gene expression was normalized to actin and compared to cells transfected with HeLa PKR knockout RNA only (mock). Data is representative of two independent experiments and error bars represent standard error of the mean.

HeLa cells were transfected with 3 μg of total RNA, and BHK21 RNA was used as the control. PKR activation at 8 hpt was determined using Western blotting. RNA from vvE3L-Rev and SPPV034/D12-S131N induced the greatest degree of PKR phosphorylation, while RNA from vvΔE3L, SPPV034 and SPPV034/D1-G801S induced lower levels of PKR phosphorylation. RNA from vvΔE3/D1-G801S and vvΔE3/D12-S131N only slightly induced PKR phosphorylation. RNA from all constructs demonstrated some level of eIF2-α phosphorylation.

PKR is integral for the detection of dsRNA during virus infections. To investigate PKR-independent responses to viral RNA species, RNA extracted from HeLa PKR knockout cell infections were transfected into HeLa PKR knockout cells as described above. Expression of cytokines IFN-β, TNF-α and IL-6 were analyzed using RT-qPCR (Figure 3.14). Although slight cytokine expression was observed in HeLa PKR knockout cell transfections, expression was severely impaired compared to standard HeLa cells. Similar to the results from HeLa cell transfections (Figure 3.13), SPPV034 RNA induced the greatest increase in expression of cytokines TNF-α and IL-6 (Figure 3.14B and C), while vvE3L-Rev RNA induced the largest increase in expression of IFN-β (Figure 3.14A). However, this increase in gene expression was not to the same magnitude as observed in HeLa cell transfections (Figure 3.13). Additionally, the correlation observed in HeLa cell transfections between dsRNA production and cytokine activation was not observed in HeLa PKR knockout cells. These results underscore the importance of PKR to the antiviral response (likely through NF-kB stimulation).

8 Vaccinia E3 protein does not co-immunoprecipitate with host ribosomal factors

Previously, it has been established that the mRNA capping enzyme of vaccinia virus also has roles as transcription factors[42]. This study provides evidence for the mRNA capping enzyme having an additional role in determining host range function of vaccinia virus. The SPPV034 recombinant virus is blocked at intermediate and late protein translation stages and mutations in the capping enzyme (D1-G801S and D12-S131N) rescue this protein translation. Next, it was investigated whether poxvirus E3 family proteins have a direct role in protein translational processes. HeLa cells were infected at an MOI of 1 with a vaccinia virus expressing either an HBH-tagged E3 protein or an HBH-tagged SPPV034 protein. Lysates were collected the

following day and analyzed using a pulldown assay with streptavidin magnetic beads and western blotting. Since the recombinant SPPV034 vaccinia virus is unable to replicate in HeLa cells, protein levels from this virus would be too low to detect interactions between SPPV034 and host proteins. Instead, this virus was used to confirm the specificity of the system for HBH-tagged E3 protein interactions.

Ten ribosomal factors were tested for interaction with vvE3 or SPPV034 protein, including RPS3, ribo-S3, phospho-S6, RPL11, RPL13A, eIF3A, eIF4A1, eIF4G1, phosphorylated eIF4E and eIF5 (Figure 3.15A). No interaction was observed between vvE3 with any of these ribosomal factors. Additionally, interactions between vvE3 protein and vvD12, eIF2α, and PKR were also analyzed. The only interaction identified was between vvE3 protein and PKR. This interaction had been previously identified by our lab[133].

Furthermore, the ability of E3 family proteins to interact directly with the m7G-cap of mRNA was investigated. HeLa PKR knockout cells were infected at MOI 1 and lysates were collected the following day. Agarose coated with m7-GTP (Creative Biomart) was used to immunoprecipitate proteins capable of interacting with the cap structure of mRNA and visualized using western blotting (Figure 3.15B). Host eIF4E is the cap-binding protein of the ribosome that facilitates translation initiation and was used as a positive control. Host eIF2α is also a ribosomal protein, but it is not associated with cap binding and therefore was used as a negative control. Both E3 and SPPV034 proteins demonstrated an affinity for the m7G-cap. Additionally, the ability of the capping enzyme D12 subunit to associate with the m7G-cap structure was investigated (since a D12 antibody was available). The pulldown demonstrated the ability of D12 to associate with m7G-cap mRNA for all viruses. It is interesting to note that while E3 protein was not able to co-immunoprecipitate with the capping enzyme D12 subunit (Figure 3.15A), both the E3 protein and the D12 subunit, were able to interact with the m7G-cap structure of mRNA (Figure 3.15B). This data indicates that E3 family proteins have an additional role associated with mRNA cap binding which is crucial for the function of E3 family proteins in determining virus host range.

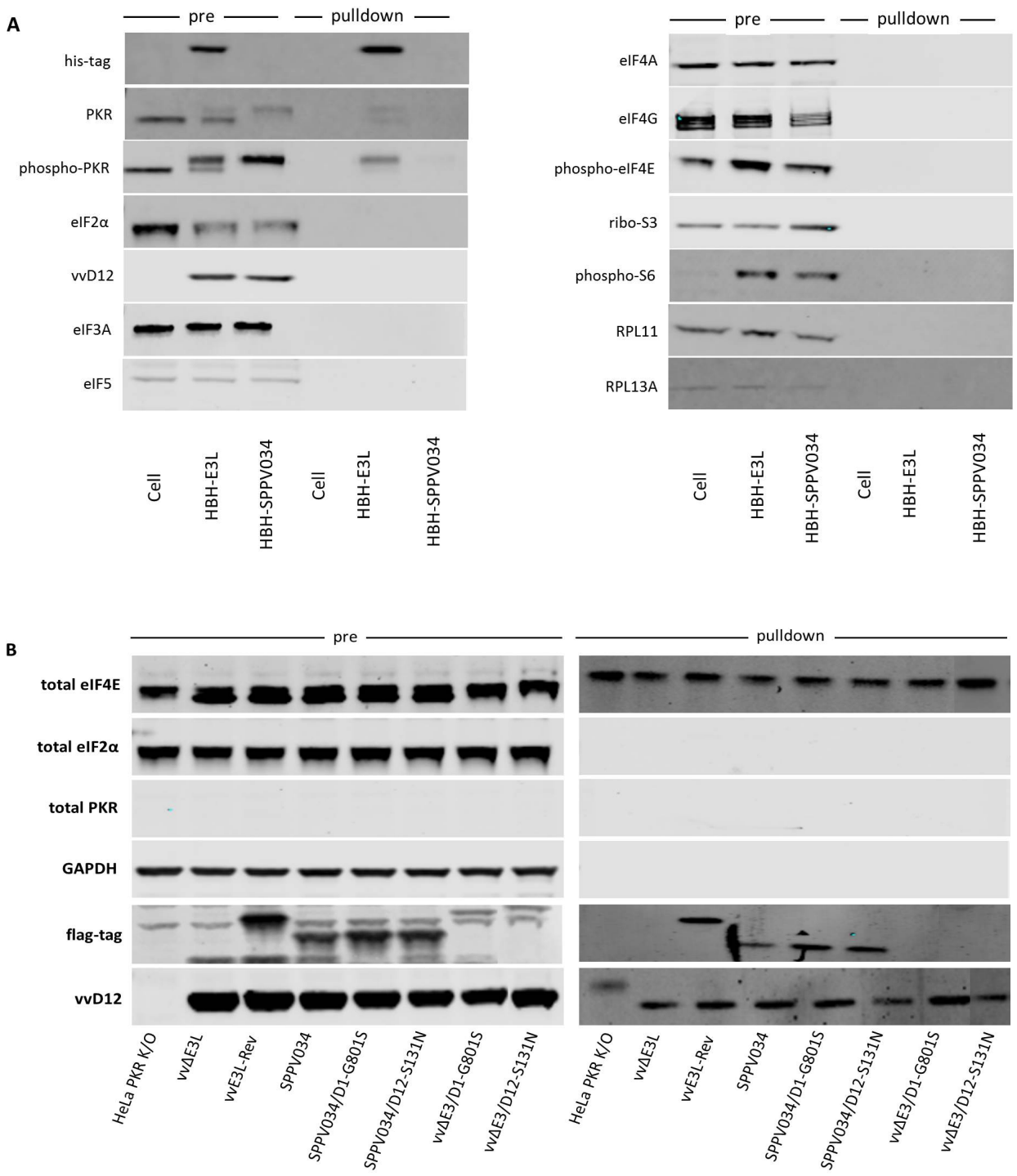

Figure 3.15. Interactions of E3 and SPPV034 proteins between host ribosomal factors and m7-GTP mRNA cap. (**A**) Ribosomal factors did not co-immunoprecipitate with E3 protein, indicated E3 family proteins do not play a role in translation. HeLa cells were infected at MOI 1 and lysed the following day. Lysate was incubated with streptavidin coated magnetic beads (Invitrogen) for 2 hours at room temperature. Interacting proteins were probed using western blotting. PKR was used as a positive interaction control. HBH-SPPV034 was used as a negative control. Actin was used as a load control. (**B**) mRNA cap interactions were investigated. Permissive HeLa PKR knockout cells were infected at MOI 1 and lysed the following day. Lysate was incubated with m7G-cap coated agarose (Creative Biomart) for 2 hours at room temperature. Interacting proteins were probed using western blotting. eIF4E was used as a positive control. PKR and GAPDH were used as a load controls.

Chapter Four: Discussion

1 Review of Background

The biochemical property of binding dsRNA has been proposed to be the basis for the biological functions of poxvirus E3 family protein. However, increasing evidence undermines this model. SPPV034 is the only E3 protein ortholog unable to rescue E3 protein functionality in a recombinant vaccinia virus, even though SPPV034 retains the ability to bind dsRNA[132]. Chimeric constructs of E3 and SPPV034 rescued virus replication in HeLa cells, which are non-permissive for the recombinant vaccinia expressing SPPV034, and demonstrated that the N-terminal Z-DNA binding domain plays an important regulatory role for this family of proteins (*Varga and Cao; manuscript in preparation*). Additionally, residues critical for dsRNA binding activity could be mutated, such that dsRNA binding was abolished, and the virus retained the ability to replicate in HeLa cells[133]. This indicates that the biochemical activity of dsRNA binding of E3 protein is not linked to its biological function for virus replication. The current study uncovered that the vaccinia virus capping enzyme, D1-D12 heterodimer, as a cofactor for the function of E3 family proteins. The D1-D12 heterodimer has also been shown to have roles in early gene transcription termination[186] and intermediate gene transcription initiation[208]. A G801S mutation in the D1 subunit and an S131N mutation in the D12 subunit were able to independently rescue replication of the SPPV034 recombinant virus.

2 Vaccinia virus mRNA capping enzyme as a cofactor for the biological function of the E3 protein

The sheeppox virus E3 ortholog SPPV034 does not function in a recombinant vaccinia virus. To investigate if there was a co-factor(s) in vaccinia virus which might be incompatible with the sheeppox virus E3 ortholog, SPPV034, EMS mutagenesis was used to induce random mutations in a recombinant vaccinia virus expressing SPPV034. Mutations associated with replication of the recombinant SPPV034 virus in HeLa cells were selected for further analysis. The EMS mutagenesis screen identified 5 potential virus cofactors, including F4, J3, D1, D11 and D12 proteins (Table 3.1). Mutations D1-G801S and D12-S131N were the only candidates capable of independently rescuing replication during single mutant virus propagation in HeLa cells.

The D1 and D12 proteins comprise the heterodimeric mRNA capping enzyme of vaccinia virus. The D1 protein is the large subunit and is organized temporally for the three catalytic reactions necessary for cap-0 formation[209–214]. The D12 protein is the small subunit, which binds to the methyltransferase domain of D1 and allosterically stimulates its activity. The D1 and D12 mutations identified in this study do not occur in residues previously identified as critical for methyltransferase or heterodimerization activities of the enzyme[170] (see vvD1 and vvD12 protein sequences in Figure 3.1).

The amino acid sequences of D1 and D12 orthologs from other poxviruses, including vaccinia virus (VACV), sheeppox virus (SPPV), monkeypox virus (MPXV), variola major (VARV), cowpox virus (CPV), ectromelia virus (EVH), camelpox virus (CMLV), molluscum contagiosum (MC), and myxoma virus (MYX; rabbitpox), were compared (Figure 3.1). The capping enzyme orthologs are highly conserved among those in the *orthopoxvirus* genus (MPXV, VAR, CPV, EVH and CMLV). Furthermore, residues previously established as critical for methyltransferase and heterodimerization activity of D1 and D12 are highly conserved among all poxvirus members. Interestingly, the mutated residues in D1 and D12 are also highly conserved among all poxvirus members. These data suggest that those residues could be under some selective pressure to remain unchanged and therefore might be crucial for the function of the enzyme.

In addition to the mRNA capping activity, vaccinia virus D1 and D12 proteins have been demonstrated to serve as an early gene transcription termination factor (usually referred to as VTF)[168,169] and an intermediate gene transcription factor (historically referred to as VITF-A)[215]. Furthermore, the capping activity of D1 and D12 was demonstrated to be independent of their role in transcription regulation[186,208]. It was reported that early gene transcription of vaccinia virus also produces dsRNA[117]. So, it is possible that the D1 and D12 mutations may affect the VTF function by reducing the amount of dsRNA produced during early stages.

Another notable finding from the EMS mutagenesis screen is that the candidates listed in Table 3.1 are all involved in virus RNA transcription and processing. The F4 protein is the small subunit of the ribonucleotide reductase. F4 is partly responsible for the rate-limiting step during dNTP synthesis and is required for efficient replication *in vitro* and *in vivo*[216,217]. Vaccinia J3 protein has been identified as the small subunit of the poly(A) polymerase, a transcription elongation factor, and the virus's (nucleoside-2'-O-) methyltransferase, which converts the mRNA cap-0 structure to cap-1[218,219]. The D11 protein is an important virus ATPase, termed

NPH1 and facilitates virus RNA polymerase read-through of transcription pause sites[188]. Although these mutations were unable to independently rescue viral replication, these factors may still have certain roles in combination with other factors. In the future, it would be interesting to investigate whether a combination of these other identified mutations can rescue replication, particularly with regards to the D12-S131N mutation.

3 The D1-G801S mutation is more influential than D12-S131N mutation in enhancing SPPV034 protein host range functionality

3.1 Mutations in the capping enzyme rescue replication of an SPPV034 recombinant virus in non-permissive cell lines

To determine how the mutations in the capping enzyme altered virus replication, virus growth kinetics were analyzed in HeLa cells. Constructs SPPV034/D1-G801S and SPPV034/D12-S131N rescued replication, while vvΔE3L, SPPV034, vvΔE3/D1-G801S and vvΔE3/D12-S131N were unable to replicate in HeLa cells (Figure 3.2A(i) and Figure 3.2B(i)). Virus yield of SPPV034/D1-G801S was similar to vvE3L-Rev, while SPPV034/D12-S131N replicated to lower levels compared to vvE3L-Rev. These data indicate that both the SPPV034 ortholog and either capping enzyme mutation (D1-G801S or D12-S131N) are necessary for the replication of the recombinant SPPV034 vaccinia virus in HeLa cells.

Aborted intermediate and late protein translation is a principal phenotype associated with defective replication of vaccinia E3L deletion mutant virus in non-permissive cells (e.g. HeLa cells). Expression of intermediate and late proteins was determined using western blotting. The constructs capable of replicating in HeLa cells (vvE3L-Rev, SPPV034/D1-G801S and SPPV034/D12-S131N) demonstrated expression of intermediate and late proteins, as observed by expression of intermediate (vvG8) and late (vvD8) proteins (Figure 3.2A(ii)) and expression correlated with the virus's replicative ability.

Protein kinase R (PKR) and RNaseL are major antiviral effectors activated by virus produced dsRNA. Both enzymes have essential, yet differing roles in innate antiviral immunity. Upon binding to dsRNA, PKR dimerizes and undergoes autophosphorylation[220]. Once activated, PKR phosphorylates eukaryotic initiation factor 2 alpha subunit (eIF2α), thereby stalling the host ribosome and preventing protein translation in infected cells. On the other hand, RNase L is activated by 2'-5' oligoadenylate synthetase (OAS). Upon recognizing foreign dsRNA, OAS oligomerizes and recruits ATP to generate 2'-5' linked oligoadenylate molecules which activate

a ribonuclease RNase L[221,222], which degrades host and viral RNAs thereby halting virus replication. Both enzymes are routine targets for viruses, several strategies to subvert these pathways have evolved[220–222]. To determine the consequences of each system to vaccinia virus replication, the ability of constructs to replicate in either HeLa PKR knockout cells or A549 PKR and/or RNaseL knockout cells was investigated.

Viral growth was examined using single-step growth kinetics in HeLa PKR knockout cells. All constructs were capable of replicating in HeLa PKR knockout cells (Figure 3.3A(i)). Since HeLa cells are defective in RNase L antiviral activity (*JXC unpublished data*), A549 cells were used to compare the abilities of the viruses to antagonize both PKR and OAS signaling arms. A549 knockout cells for PKR, RNAseL or both PKR and RNAseL were used to examine virus replication via plaque formation and protein expression. Plaque formation was monitored using fluorescent microscopy, and the detection of either a red or green fluorescent marker cloned into all viruses (see Materials and Methods). Viruses vvE3L-Rev and SPPV034/D1-G801S were capable of replicating in all A549 cell lines (Figure 3.3B).

Conversely, the SPPV034/D12-S131N virus demonstrated restricted plaque formation and protein expression in A549 wild-type and RNAseL knockout cell lines. These data indicate that the combination of the mutation in D12L and SPPV034 is not sufficient to antagonize PKR while the mutation in D1R and SPPV034 can. Although vvΔE3L and SPPV034 viruses demonstrated slight plaque formation in A549 PKR knockout cells, recent virus yield assays demonstrate virus growth above baseline occurs in only A549 double-knockout cells (data not shown). These experiments indicate that a combination of SPPV034 and a capping enzyme mutation (D1-G801S or D12-S131N) are required to antagonize both PKR and RNAseL pathways.

3.2 The mutation in the D1 subunit influences host innate immune modulation mechanisms of SPPV034 recombinant virus

To investigate how the mutations in the capping enzyme collaborate with SPPV034 to influence host immune responses, the activation of PKR and eIF2α was examined using western blotting. Constructs vvE3L-Rev and SPPV034/D1-G801S inhibited PKR and eIF2α phosphorylation (Figure 3.4A), while SPPV034 with the wild-type D1R, could not. While SPPV034/D12-S131N could not inhibit PKR phosphorylation to the same degree as SPPV034/D1-G801S, the level of phosphorylation was slightly decreased compared to SPPV034 (which has the wild-type D1R). Constructs vvΔE3/D1-G801S and vvΔE3/D12-S131N were also

unable to inhibit PKR activation. These data indicate that both the SPPV034 and the mutated D1 protein are required to inhibit PKR activation.

In comparison to vvΔE3L, the vvΔE3L/D1-G801S induced considerably less PKR phosphorylation, while vvΔE3/D12-S131N induced slightly less PKR activation. This further suggests that the biological phenotypes (e.g. virus replication in HeLa and PKR phosphorylation) seen from the SPPV034/D1-G801S is the result of the additive effects of both SPPV034 and the mutated D1 protein. This is further supported by the levels of PKR phosphorylation observed at 5hpi (Figure 3.4A). While vvΔE3L induced a considerable level of PKR phosphorylation at 5hpi, SPPV034 induced noticeably lower levels of PKR phosphorylation. This suggests that SPPV034 retains partial ability to inhibit PKR activation at least at early time points. This also suggests that the mutations in the capping enzyme (D1-G801S and D12-S131N) also play an individual role in inhibiting PKR activation, possibly through altered dsRNA levels produced by these constructs (see section 4.2 for further discussion).

SPPV034/D12-S131N was not able to inhibit PKR phosphorylation to the same degree as SPPV034/D1-G801S. However, this mutation was still able to rescue replication in HeLa cells (Figure 3.2A(i) and Figure 3.2B(i)). Interestingly, vvΔE3L/D1-G801S induced less PKR phosphorylation than SPPV034/D12-S131N but remains unable to replicate in HeLa cells. However, this could be due to lower levels of dsRNA produced by vvΔE3/D1-G801S (Figure 3.9A(ii)). Other vaccinia constructs have been identified that are able to replicate in HeLa cells in the presence of activated PKR (*Varga and Cao; manuscript in preparation*). Taken together, these data suggest that vaccinia virus has another factor(s) collaborating with the E3 family proteins to antagonize the antiviral state induced by phosphorylation of PKR. Alternatively, the proportion of activated PKR could be critical.

It has been observed that the early detection of viral infection and the early activation of host defenses (such as PKR phosphorylation/activation) is paramount for an effective antiviral response. PKR phosphorylation was prominently detected at 5 hpi in vvΔE3L infection (Figure 3.4A). To further characterize this phenotype, HeLa cells were treated with or without cytosine arabinoside (ara-C) to block vaccinia genome replication and therefore transcription of intermediate/late genes (the primary source of viral dsRNA). Treatment of HeLa cells with ara-C drastically reduced PKR phosphorylation levels in all infections except vvΔE3L (Figure 3.4B). These data indicate that vaccinia virus early gene by-products are also capable of activating the PKR pathway and that E3 family proteins have an important role in preventing their detection.

Interestingly, SPPV034 retains the ability to inhibit PKR phosphorylation during early stages of infection, indicating that it is able to interact with early virus factors/products that activate PKR (compare vvΔE3L and SPPV034 lanes in Figure 3.4B, phospho-PKR with ara-C treatment).

Apoptosis is another major antiviral mechanism. Poly (ADP-ribose) polymerase (PARP) is a family of post-translational modification proteins. These proteins are crucial elements in DNA repair mechanisms[223]. Under apoptotic conditions, PARP is cleaved by caspases thereby preventing DNA repair. Increases in DNA damage leads to apoptosis. Caspase-3 and caspase-7 have been implicated as the enzymes for PARP cleavage. Furthermore, caspase-7 is one of the final effector caspases in the cascade leading to programmed cell death[224]. Both PARP cleavage and caspase cascade initiation have been associated with PKR activation[225].

The activation of apoptotic signaling proteins PARP and caspase 7 were also measured using western blotting (Figure 3.5). Constructs vvE3L-Rev, SPPV034/D1-G801S and SPPV034/D12-S131N inhibited cleavage of PARP and caspase 7. These data indicate that SPPV034/D12-S131N inhibits apoptosis downstream of PKR activation since SPPV034/D12-S131N could not efficiently inhibit PKR activation. Inhibition of these apoptotic markers is not as critical for vaccinia virus replication as PKR since constructs that demonstrate cleavage of PARP and caspase 7 are also still able to replicate to considerable titres in HeLa cells (*Varga and Cao; manuscript in preparation*). So far, six vaccinia proteins have been described as having anti-apoptotic functions including B13, B22, F1, N1, M1 and E3 protein[226]. However, proteins B22, F1 and E3 were also shown to have no effects on apoptosis in infections with the modified Ankara strain of vaccinia virus (MVA). These data indicate that the role of E3 protein in inhibiting apoptosis may depend on an association with those other viral factors, instead of acting in an independent and redundant manner.

3.3 The mutation in the D1 subunit collaborates with SPPV034 to suppress cytokine expression

To determine if the mutations in the capping enzyme were associated with host antiviral cytokine response, the expression of cytokines IFN-β, TNF-α and IL-6 were determined using quantitative real-time PCR (qPCR). Construct SPPV034/D1-G801S suppressed cytokine expression to levels similar to vvE3L-Rev (Figure 3.6). These data indicate that the mutation in D1 can influence the cytokine expression during virus infection. Construct vvΔE3/D1-G801S also induced less expression of IFN-β, although not to the same level as SPPV034/D1-G801S. These data support that the immunomodulatory effects of the virus are due to the combined

effects of the mutation in D1R and SPPV034. Interestingly, it is noted that the mutation D12-S131N does not influence cytokine expression in a similar manner as the D1-G801S mutation.

Peculiarly, constructs vvΔE3/D1-G801S and vvΔE3/D12-S131N demonstrated cytokine expression in a similar manner as SPPV034. Based on previous data from our lab, the SPPV034 ortholog, under the control of a native E3 promoter, retains the ability to modulate cytokine expression (*Varga and Cao; manuscript in preparation*). This was the only immunomodulatory function identified for this ortholog when expressed in a recombinant vaccinia virus. It was expected that the removal of the ortholog would result in the constructs vvΔE3/D1-G801S and vvΔE3/D12-S131N behaving similarly to vvΔE3L. However, vvΔE3/D1-G801S and vvΔE3/D12-S131N behaved more similar to SPPV034 in regards to cytokine levels than vvΔE3L. The mutated capping enzyme may affect cytokine response through the combined effects of an altered PAMP (dsRNA) and related signal pathways (see the dot-blot section for discussion).

It was found that expression of all three cytokines examined was noticeably reduced in HeLa PKR knockout cells compared to standard HeLa cells (Figure 3.7). Expression of IFN-β remained relatively unchanged for all constructs compared to uninfected cells. PKR is a main dsRNA pattern recognition receptor (PRR), mainly via signaling to downstream transcription factors including NFkB and other IRFs[225]. Without this crucial initiator, the expression of IFN-β is radically impacted.

PKR activation has been shown to be strongly correlated with TNF-α expression[204,227]. The importance of PKR in TNF-α expression is underscored in that expression of TNF-α was reduced approximately 275-fold in vvΔE3L infection in HeLa PKR knockout cells compared to vvΔE3L infection in standard HeLa cells (Figure 3.6B and Figure 3.7B). Furthermore, expression of IL-6 was marginally increased following infection in HeLa PKR knockout cells. IL-6 is not directly involved in dsRNA detection or signaling but instead is stimulated by a number of different ‘emergency' detection pathways (including infections and tissue damage)[228]. Like TNF-α, the IL-6 expression is also regulated by NFkB signaling. Therefore the slight increase in IL-6 expression observed in HeLa PKR knockout cells is likely a result of other PKR-independent NFkB signaling pathways. Nonetheless, PKR activation appears to have a key role in all host defense mechanisms, as the expression of IL-6 was reduced approximately 80-fold in vvΔE3L infection in HeLa PKR knockout cells compared to vvΔE3L infection in standard HeLa cells (Figure 3.6C and Figure 3.7C).

4 Biochemical property of dsRNA affects E3 family protein function

4.1 Localization of dsRNA and E3 family proteins during infection

To determine whether the capping enzyme mutations would alter the cellular distribution of dsRNA, immunofluorescent confocal microscopy was used to visualize dsRNA and vvE3 or SPPV034 protein within infected cells. All constructs produced a detectable amount of dsRNA and E3 or SPPV034 (where present) at 5 hpi (Figure 3.8A). However, constructs capable of replicating in HeLa cells (vvE3L-Rev, SPPV034/D1-G801S and SPPV034/D12-S131N) demonstrated an increase in dsRNA over time compared to non-replicating constructs (Figure 3.8A, 12hpi pane). All constructs displayed strong colocalization between vvE3 or SPPV034 protein and dsRNA expression at early and late times. These data indicate that the mutations in D1R or D12L do not alter dsRNA distribution during infection.

Although there is evidence that the recombinant SPPV034 virus retains the ability to bind dsRNA in a poly I:C pulldown assay[132] (*Varga and Cao; manuscript in preparation*), this interaction has never been demonstrated during an infection. One concern with the SPPV034 recombinant virus's inability to replicate in HeLa cells was that the SPPV034 protein was unable to interact with vaccinia virus dsRNA within the host cell due to structural differences between SPPV034 and E3. Another possibility was that viral dsRNA and SPPV034 was not able to interact as they were not being expressed in the same location. As shown in Figure 3.8A, SPPV034 strongly co-localizes with dsRNA. However, the expression of dsRNA and SPPV034 is diminished at late times compared to replicating constructs SPPV034/D1-G801S and SPPV034/D12-S131N. Therefore, the inability of the recombinant virus expressing SPPV034 to replicate in HeLa cells is not due to a failure of SPPV034 to co-localize with dsRNA during infection. Nor is the deficient replication due to dsRNA and SPPV034 localizing to different areas of the infected cell. Instead, the reduced levels of SPPV034 and dsRNA during infection indicate the recombinant virus fails to overcome early host defenses and make the switch to intermediate/late gene expression (as dsRNA is largely a by-product of intermediate and late transcription post-genome replication). Treatment with Ara-C greatly reduced the amount of dsRNA produced by replicating viruses, but co-localization of dsRNA and the virus dsRNA binding protein was unaffected.

Since SPPV034 virus does not replicate in standard HeLa cells, HeLa PKR knockout cells were used to compare the co-localization of SPPV034 and E3 proteins with late viral

dsRNA. All constructs demonstrated increases in dsRNA over time in these cells (Figure 3.8B). Co-localization of dsRNA and E3 or SPPV034 proteins also occurred in aggresome type structures similar to infections in HeLa cells. These data indicate that SPPV034 and E3 proteins co-localize with late dsRNA products in a similar manner, irrespective of the D1 or D12 mutations.

4.2 Host cell influences the dsRNA quantity produced during infection

To determine the differences in the quantity of dsRNA produced from each construct, dsRNA was quantified using a dot-blot with the dsRNA J2 antibody. It has been observed that the J2 antibody could partially recognize host dsRNA products such as tRNA and rRNA[229]; therefore the dsRNA signal was normalized using uninfected cells. Constructs capable of replicating in HeLa cells (vvE3L-Rev, SPPV034/D1-G801S and SPPV034/D12-S131N) demonstrated considerable amounts of dsRNA after 12 hours (Figure 3.9A(ii)). SPPV034/D12-S131N produced approximately 2-fold less dsRNA than vvE3L-Rev or SPPV034/D1-G801S. These data suggest that the bulk of vaccinia virus dsRNA is produced during intermediate/late gene expression. Replicating viruses vvE3L-Rev, SPPV034/D1-G801S and SPPV034/D12-S131N demonstrated approximately 18-fold, 11-fold and 12-fold increases in dsRNA signal intensity, respectively, compared to dsRNA levels from early infection stages (compare Figure 3.9A(ii) and Figure 3.9B(ii)). Whereas non-replicating viruses vvΔE3L, SPPV034, vvΔD1/G801S and vvΔE3/D12-S131N demonstrated 3-fold, 5-fold, 2-fold and 5-fold increases in dsRNA signal intensity, respectively. The replicative capability of the virus can also be correlated to the amount of dsRNA produced during infection as SPPV034/D12-S131N demonstrated reduced dsRNA production and replicated at a slower rate compared to vvE3L-Rev and SPPV034/D1-G801S (compare Figure 3.2A(i) and Figure 3.9A(ii)). These results indicate that replicating viruses contain a mechanism to prevent dsRNA detection pathways of the host from activating as vvE3L-Rev and SPPV034/D1-G801S produce significant amounts of dsRNA without activating PKR (compare Figure 3.4A and Figure 3.9A(ii)).

Early gene transcription termination activity of the capping enzyme can be inferred from dsRNA levels produced early in infection. Since early transcripts have a definitive termination sequence, the 3’ ends are unlikely to overlap with transcripts from adjacent early genes. It has been established that abolishing the termination activity of the vaccinia virus capping enzyme results in early transcripts that are longer and induce host antiviral responses[186]. Ara-C was used to determine the effects of the D1 and D12 mutations on termination activity for early gene

transcription. Ara-C blocks vaccinia genome replication, therefore any RNA transcripts following ara-C treatment will be from early transcription. Ara-C treatment reduced dsRNA production in all constructs to relatively similar levels (Figure 3.9B(ii)), indicating that the termination activity of the capping enzyme was not altered due to the mutations in D1R or D12L. These data also indicate that all constructs are capable of minor levels of dsRNA production during the early stage of infection. It has been recently established that VACV K1 protein is an important regulator of host responses during early infection stages[230].

To further investigate the impact of the D1R and D12L mutation on total dsRNA production, HeLa PKR knockout cells and BHK21 cells were infected (as all viruses replicate comparatively in these cells). dsRNA was quantified using the J2 antibody as described above. In PKR knockout cells (Figure 3.10A(i)), SPPV034 produced the largest amount of dsRNA, while vvE3L-Rev, SPPV034/D1-G801S, SPPV034/D12-S131N produced moderate levels of dsRNA and vvΔE3L, vvΔE3/D1-G801S and vvΔE3/D12-S131N demonstrated the lowest dsRNA levels at 12hpi (Figure 3.10A(ii)). In HeLa PKR knockout cells the mutations in the capping enzyme reduce the amount of dsRNA albeit to different degrees. The D12-S131N mutation slightly reduces dsRNA produced from SPPV034/D12-S131N compared to SPPV034 and vvΔE3/D12-S131N compared to vvΔE3L. Whereas the D1-G801S mutation greatly reduces the amount of dsRNA produced from SPPV034/D1-G801S compared to SPPV034 and vvΔE3/D1-G801S compared to vvΔE3L.

In BHK21 cells, however, SPPV034/D12-S131N produced the largest amount of dsRNA and vvΔE3L, vvE3L-Rev, SPPV034 and SPPV034/D1-G801S produced moderate amounts (Figure 3.11A(ii)). Whereas, vvΔE3/D1-G801S and vvΔE3/D12-S131N produced the lowest levels of dsRNA. In BHK21 cell infections, the capping enzyme mutations did not result in reduced dsRNA amounts like in HeLa PKR knockout cell infections (compare 12hpi-DB-BHK to 12hpi-DB-PKRko). When compared to vvΔE3L, vvΔE3/D12-S131N demonstrated reduced levels of dsRNA and vvΔE3/D1-G801S demonstrated a greater reduction in dsRNA levels. This is a similar result to infections in HeLa PKR knockout cells. However, the capping enzyme mutations increased dsRNA levels in SPPV034/D12-S131N and SPPV034/D1-G801S compared to SPPV034. In other words, the D1-G801S mutation slightly increased dsRNA levels and the D12-S131N mutation greatly increased dsRNA levels in the presence of the SPPV034. These data suggest that dsRNA levels are cell line dependent and potentially a cellular factor(s) influence the amount of dsRNA produced during virus infection.

Likewise, treatment with ara-C reduced dsRNA levels in all constructs in both HeLa PKR knockout and BHK21 infections (Figure 3.10B(ii) and Figure 3.11B(ii)). It was noted that after normalization to uninfected cells, the quantity of dsRNA from vvΔE3L and vvΔE3/D12-S131N infections in BHK21 cells resulted in negative values (Figure 3.11B(ii)). Since vaccinia virus infection results in shut down of host transcription processes, including tRNA production[231], these infections could result in less cellular dsRNA products thereby generating negative values. This is the first demonstration that differing amounts of a PAMP can be produced in different host cells. Since different host cells will contain varying immune system compositions, the virus-host tropism is determined by the interplay of both virus and host factors.

4.3 Vaccinia mRNA capping enzyme has a role in influencing viral dsRNA PAMPs and the related antiviral responses

To determine the effects of the capping enzyme mutations on the ability of dsRNA to induce host innate immune responses, HeLa cells were transfected with RNA harvested from infections in HeLa PKR knockout cells. Late RNA from all constructs induced PKR and eIF2α phosphorylation, while RNA from HeLa PKR knockout cells only did not (Figure 3.12A). The level of phosphorylation of PKR strongly correlated with the amount of dsRNA each construct produced (compare with Figure 3.10A(ii)), in that SPPV034 induced the largest amount of phosphorylation. Whereas vvΔE3L, vvE3L-Rev, SPPV034/D1-G801S and SPPV034/D12-S131N induced moderate amounts of phosphorylation and vvΔE3/D1-G801S and vvΔE3/D12-S131N induced extremely low levels. Since the amount of RNA used for transfection was taken as a measurement of total RNA within extract samples, it could be that the amount of dsRNA structures within that extract sample varied between constructs. For example, SPPV034 produced the most dsRNA during infections in HeLa PKR knockout cells (Figure 3.10A(ii)) and induced the largest degree of PKR phosphorylation upon RNA transfection in standard HeLa cells (Figure 3.12A). Whereas the infection with vvΔE3/D1-G801S produced the least amount of dsRNA and induced very little PKR phosphorylation. This pattern was also observed when looking at cytokine expression post-transfection (Figure 3.13). These data strongly indicate that the mutations in the mRNA capping enzyme influence the amount of dsRNA produced during virus infection. SPPV034/D12-S131N demonstrated a slight decrease in PKR phosphorylation, while SPPV034/D1-G801S demonstrated a larger decrease in PKR phosphorylation when compared to SPPV034 (which contains the wild-type capping enzyme). Furthermore, vvΔE3/D12-S131N also demonstrated a slight decrease in PKR phosphorylation, while

vvΔE3/D1-G801S demonstrated a larger decrease in phosphorylation compared to vvΔE3L (which also contains the wild-type capping enzyme).

Another possibility is that a property of viral RNA is altered during infection because of the differing components/mutations in each construct. This change to a biochemical property of the viral RNA would induce a different antiviral response compared to extracts from other constructs. This is evident from transfections using RNA from BHK21 cell infections as the degree of PKR phosphorylation did not correlate with the amount of dsRNA produced from similar cell infections (Figure 3.11A(ii) and Figure 3.12B). In BHK21 cell infections, SPPV034/D12-S131N produced the most dsRNA and transfections using RNA from SPPV034/D12-S131N demonstrated the largest degree of PKR phosphorylation. Interestingly, constructs vvE3L-Rev and SPPV034/D1-G801S demonstrated similar dsRNA levels, however transfections using RNA from vvE3L-Rev induced considerably larger amounts of PKR phosphorylation than SPPV034/D1-G801S, which displayed similar levels of PKR phosphorylation as the other constructs (compare Figure 3.11A(ii) and Figure 3.12B). Transfections using RNA from vvΔE3/D1-G801S and vvΔE3/D12-S131N infections induced the lowest degree of PKR phosphorylation, which correlates with the lower dsRNA levels detected in the dot-blot section. These data indicate that the combination of SPPV034 and the capping enzyme subunit D1 or D12 may affect both the quantity and biochemical properties of the viral dsRNA population.

5 E3 family proteins do not appear to interact with host ribosomal factors

Recombinant vaccinia viruses vvΔE3L and SPPV034 do not replicate in HeLa cells as these viruses are blocked from translating intermediate and late proteins. Mutations in the D1 or D12 subunit of the mRNA capping enzyme aids the SPPV034 recombinant virus in overcoming this blockade. This investigation provides evidence that the mutations may also alter the biochemical nature of dsRNA produced during vaccinia virus infection in D1 or D12 subunits. Furthermore, for the virus to replicate both the SPPV034 protein and capping enzyme mutation (either D1-G801S or D12-S131N) are required. Therefore, it was proposed that SPPV034 plays a direct role in protein translation processes. Interactions between E3 or SPPV034 and host protein translation machinery were investigated using co-immunoprecipitation assays.

Since a critical function of the D1-D12 heterodimer is the proper capping of vaccinia virus mRNA transcripts, it was possible that the E3 ortholog, SPPV034, may require an altered cap structure for proper recognition of RNA transcripts and subsequent translation initiation. To investigate if E3 family proteins have a role as translation factors, a co-immunoprecipitation assay was used to examine if there was an interaction between E3 family proteins and host ribosomal factors. The infected HeLa cell lysates were incubated with streptavidin-coated magnetic beads to isolate E3 or SPPV034 with a biotin tag. Potential interacting ribosomal factors were probed using western blotting. E3 protein did not co-immunoprecipitate with any ribosomal factors examined (Figure 3.15A). Previous studies have shown that E3 co-immunoprecipitates with PKR[133], which was used in this study as a positive control. Furthermore, E3 also did not co-immunoprecipitate with the small subunit of the capping enzyme D12. Still, E3 family proteins may be acting as translation factors without direct interaction with ribosomal factors.

To further investigate if E3 family proteins are involved in recognition of the mRNA cap, a pulldown assay using agarose beads coated with m7-GTP to isolate factors capable of binding to the mRNA cap. Host factors eIF4E and eIF2α, and virus proteins E3 or SPPV034 and D12 were probed using western blotting (Figure 3.15B). Host ribosome subunit eIF4E is responsible for binding the mRNA cap and recruiting other translation factors[148]. This factor was used as a positive control. The eIF2α subunit is also involved in regulating translation but does not bind to the mRNA cap. This factor was used as a negative control. The VACV capping enzyme subunit D12 was isolated from all constructs, indicating that neither mutation affected the D1-D12 heterodimer's ability to bind the mRNA cap. Interestingly, E3 and SPPV034 were also able to bind the mRNA cap. This is the first demonstration that E3 family proteins are capable of binding to an m7-GTP cap structure. Further investigations into the mechanism and purpose of this interaction will be necessary to elucidate the importance of this interaction for host tropism and viral replication.

Investigations using human cytomegalovirus (HCMV) TRS1 protein has yielded similar results and implies a similar mechanism. TRS1 protein is a known antagonist of PKR and has been demonstrated to have both PKR and RNA binding motifs[232–234]. Additionally, researchers have used HCMV TRS1 to rescue an E3-deficient vaccinia virus[232], indicating these proteins have similar functions. For example, TRS1 has also been shown to inhibit PKR activation and binds to PKR directly[232–234]. Recently, Ziehr *et al* (2015) demonstrated that TRS1 is also capable

of binding the m7-cap of mRNA transcripts[233]. This binding co-localized with mRNAs undergoing translation initiation, increased protein translation and preferentially bound viral 5'-UTR's. In the future, it would be interesting to examine if E3 family proteins displayed a similar phenotype during vaccinia virus infections.

Chapter Five: Conclusions

In this study, we further investigated the previous finding that the E3 ortholog from sheeppox virus, SPPV034, was not able to restore virus host range in a recombinant vaccinia virus[132]. SPPV034 retained the ability to bind dsRNA, however, it failed to inhibit various host innate immune responses including activation of PKR and eIF2α (which are essential in the detection of the viral PAMP dsRNA). It was hypothesized that SPPV034 was incompatible with a vaccinia virus derived cofactor(s) and therefore this interaction is essential for E3 family protein role in determining virus host range. EMS mutagenesis identified that the capping enzyme of vaccinia virus (a heterodimer of the D1 and D12 proteins) could collaborate with SPPV034 to determine virus host range. The D1-G801S mutation rescued virus replication in HeLa cells (Figure 3.2A(i) and Figure 3.2B(i)) and fully inhibited host innate immune responses including activation of PKR, inhibited apoptosis (Figure 3.4A and Figure 3.5), and suppressed IFN-β expression (Figure 3.6A). The D12-S131N mutation was able to partially rescue replication in HeLa cells (Figure 3.2A(i) and Figure 3.2B(i)). However, this virus was not able to fully antagonize aspects of the host innate immune response (Figure 3.4, Figure 3.5 and Figure 3.6). RNA quantification analysis revealed that the D1-G801S and D12-S131N reduced dsRNA in infections in HeLa PKR knockout cells (Figure 3.10). The D12-S131N mutation slightly reduced dsRNA levels, whereas the D1-G801S mutation considerably reduced dsRNA levels. This indicates a novel function of the mRNA capping enzyme in regulating dsRNA produced during virus replication. In addition to mRNA capping, the D1-D12 heterodimer has been previously shown to have roles in early transcription termination and intermediate transcription initiation[186,208]. Whether these transcription regulation mechanisms are also involved in regulating dsRNA levels remains to be further investigated.

From this study, we propose that E3 family proteins operate on a threshold mechanism and intrinsic properties of each protein determine this threshold. SPPV034 protein can inhibit PKR activation during early stages of infection (Figure 3.4A), although this activity is not as potent as E3 protein. In contrast, vvΔE3L, which lacks either SPPV034 or E3 protein, demonstrated significant levels of phosphorylated PKR at 5 hpi. At late stages, however, more virus PAMPs (primarily dsRNA) are produced from intermediate/late gene transcription. dsRNA levels would then exceed the optimal threshold for SPPV034 protein function, and its ability to contain virus PAMPs is overwhelmed, thereby resulting in host response pathway activation. E3

protein maintains the ability to control virus PAMP detection much later in infection, as slight PKR activation is observed at 10 hpi (Figure 3.4B). Progeny virus packaging has already begun at this stage, therefore antiviral responses are not effective at curbing viral replication.

During infections with the D1-G801S or D12-S131N mutant viruses, the quantity and/or a property of a viral dsRNA (or a different viral PAMP altogether) is lower and does not reach the upper limit for SPPV034 protein function. Thus, these viruses are capable of replicating in HeLa cells. This threshold mechanism will also explain the differences in replicative ability of the D1-G801S and D12-S131N mutant viruses. The SPPV034/D1-G801S virus replicates slower than vvE3L-Rev (Figure 3.2B(i)). This virus also produces considerably less dsRNA compared to SPPV034 (Figure 3.10). Therefore it is likely that, in the SPPV034/D1-G801S infection, the SPPV034 was antagonizing the viral PAMP induced antiviral response at a slower or less efficient manner than vaccinia E3 protein. A similar explanation may apply to the SPPV034/D12-S131N virus, which replicates considerably slower than vvE3L-Rev or SPPV034/D1-G801S (Figure 3.2A(i) and Figure 3.2B(i)). Viruses vvΔE3/D1-G801S and vvΔE3/D12-S131N are not able to replicate (Figure 3.2A(i) and Figure 3.2B(i)) in HeLa cells, even though they produce substantially lower levels of dsRNA compared to all constructs (Figure 3.9, Figure 3.10 and Figure 3.11). These viruses lack SPPV034 or E3 proteins, which would neutralize the antiviral activities induced by the low level of dsRNA. Moss *et al.* (2015) recently demonstrated the importance of efficient dsRNA turnover for VACV replication[235]. This trend is not observed in BHK21 cell infections as the viruses in this study replicate to comparable levels in this cell line and dsRNA levels vary drastically between constructs. A possible explanation for this is that these two cell lines (HeLa vs BHK21) will have significantly different innate immune compositions and thus different responses to virus infection. Consequently, the different cellular context may relax specific host constraints and allow the virus to utilize different strategies to antagonize host antiviral responses.

This study outlines the complicated nature of the virus-host interaction and that there is a delicate balance in the amount of dsRNA produced during VACV replication. The virus has to produce enough RNA to allow for sufficient transcript levels for structural protein synthesis (encoded by intermediate genes) and early transcription factors for subsequent infections (encoded by late genes). Due to the nature of VACV transcription processes, intermediate/late gene transcription results in run-through transcripts and thus large amounts of dsRNA. To compensate, the virus utilizes a dsRNA binding protein (E3 family proteins) which is essential

for the inhibition of host innate immune system activation. However, poxviruses need to control the levels of dsRNA so not to overwhelm the antagonizing capacity of the E3 family proteins (as is the case when SPPV034 protein is substituted for E3 in VACV). Therefore it is a combined effort of the viral antagonist of host innate immune system (E3 family proteins), viral PAMP which can stimulate host innate antiviral response (dsRNA) and host innate antiviral immunity (PKR activation, cytokine expression, etc) that determine the outcome of virus replication in a particular host.

Chapter Six: References

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Chapter Seven: Appendix

Table A 1. Summary of Vaccinia Virus Recombinants generated and used in this study

Virus Name	Vaccinia Strain	E3L Gene Status	Transgene	Mutation	Fluorescent Protein Gene
vvΔE3L	Western Reserve	–	–	–	dsRED
vvE3L-Rev	Western Reserve	+	–	–	dsRED
SPPV034	Western Reserve	–	SPPV034L	–	dsRED
SPPV034/D1-G801S	Western Reserve	–	SPPV034L	+ (G801S)	dsRED
SPPV034/D12-S131N	Western Reserve	–	SPPV034L	+ (S131N)	dsRED
vvΔE3/D1-G801S	Western Reserve	–	–	+ (G801S)	EGFP
vvΔE3/D12-S131N	Western Reserve	–	–	+ (S131N)	EGFP

Table A 2. Real-time PCR primer/probe sequences

Real time PCR primer/probes	Sequences (5'-3')
Actin	For: CACACTGTGCCCATCTACGA
	Rev: GCCAGCCAGGTCCAGAC
	Probe: CCCATGCCATCCTGC
IFNβ	For: TGGCTGGAATGAGACTATTGTTGAG
	Rev: CAGGACTGTCTTCAGATGGTTTATCT
	Probe: CCTCCTGGCTAATGTC
IL-6	For: AGATGGATGCTTCCAATCTGGATTC
	Rev: TCAAACTCCAAAAGACCAGTGATGA
	Probe: ACCAGGCAAGTCTCCTCA
TNFα	For: GCCCCTCCACCCATGTG
	Rev: GGTTGACCTTGGTCTGGTAGGA
	Probe: ACCCACACCATCAGCC

Table A 3. Summary of antibodies used in this study

Antibody Name	Dilution Used	Company	Cat No.
PKR [YE350]	1:1000 WB	abcam	ab32052
PKR [E120] phospho-Thr446	1:1000 WB	abcam	ab32036
eIF2 [E90] phospho -Ser51	1:1000 WB	abcam	ab32157
eIF2	1:1000 WB	Invitrogen	AHO1182
actin	1:5000 WB	abcam	ab46805
vvD8	1:1000 WB	gift from Yan Xiang; San Antonio HSC, UT	
vvD12	1:1000 WB	Genscript	custom peptide
vvG8	1:1000 WB	Genscript	custom peptide
PARP, cleaved (Asp214) [E2T4K]	1:1000 WB	Cell Signaling Technologies	32563S
caspase 7, cleaved (Asp198)	1:1000 WB	Cell Signaling Technologies	9491S
M2 Flag	1:1000 WB 1:100 IFA	Sigma	F3165-1MG
J2 (dsRNA)	1:1000 WB 1:100 IFA 1:1000 dot-blot	Scions	
GAPDH	1:1000 WB	Epitomics	2251-1
eIF4G1	1:1000 WB	abcam	ab47649
eIF4A1 [EPR14506]	1:1000 WB	abcam	ab185946
eIF5	1:1000 WB	abcam	ab228874
eIF4E [Y448]	1:1000 WB	abcam	ab33766
phospho-eIF4E (Ser209) [EP2151Y]	1:1000 WB	abcam	ab76256
ribosomal protein S3 (D50G7)	1:1000 WB	Cell Signaling Technologies	9538S
RPL11 (D1P5N)	1:1000 WB	Cell Signaling Technologies	18163S
eIF3A (D51F4)	1:1000 WB	Cell Signaling Technologies	3411S
phopho-ribosomal protein S6 (Ser235/Ser236)	1:1000 WB	Invitrogen	44-922G
RPL13A	1:1000 WB	Invitrogen	PA5-17176
RPS3	1:1000 WB	Invitrogen	PA5-17214

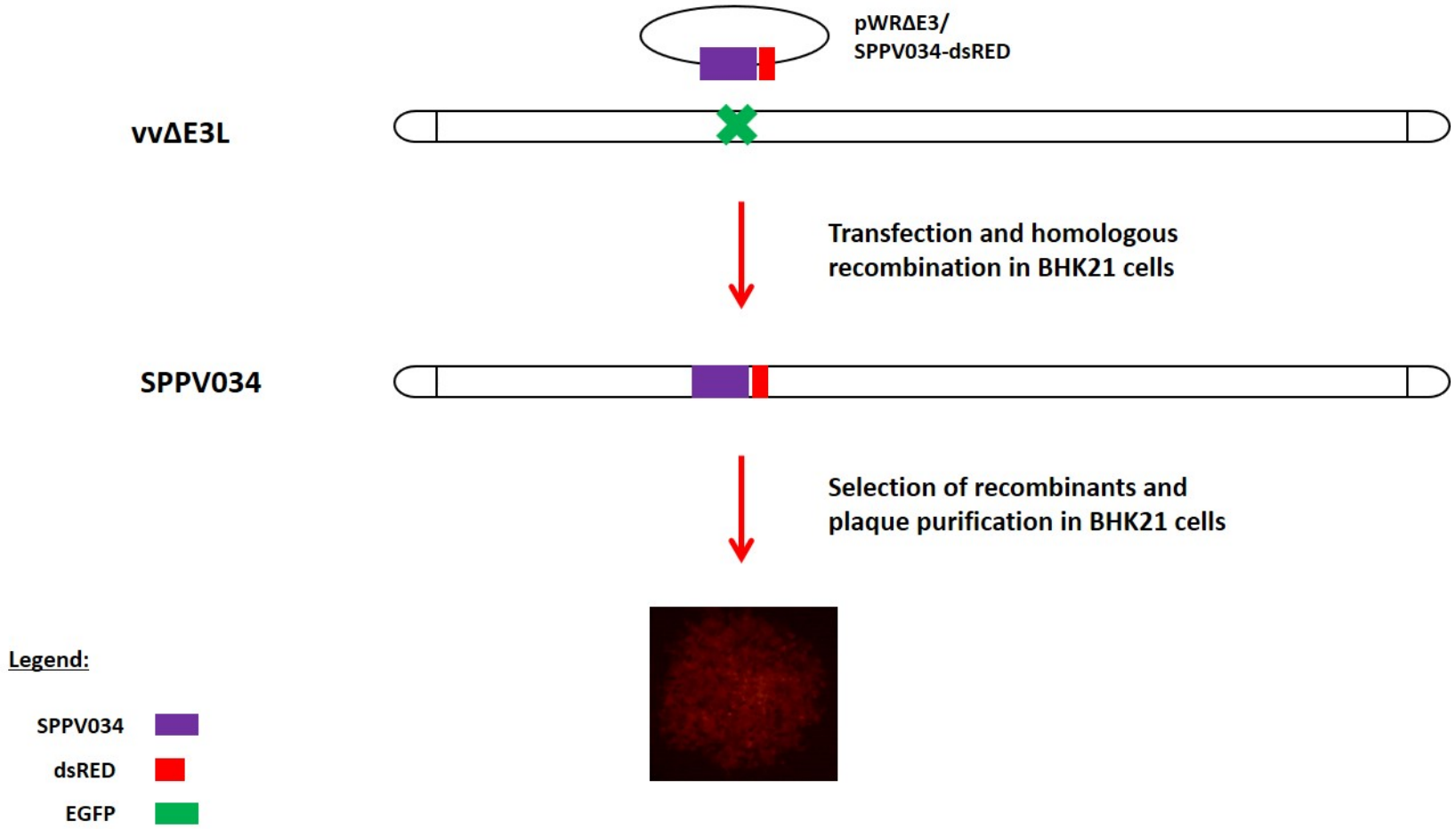

Figure A 1. Schematic of the generation of the SPPV034 recombinant virus.
The SPPV034L gene from sheeppox virus was cloned in between left and right flanking regions, which corresponds to 600 bps of identical sequences flanking the E3L locus of vaccinia virus. A dsRED fluorescent protein was used to facilitate selection and purification. The resulting pWRΔE3/SPPV034-dsRED vector was transfected into BHK21 cells infected with an E3-knockout virus expressing EGFP. Double crossover events were selected via the detection of red only virus plaques and recombinant virus was purified using three rounds of plaque purification.

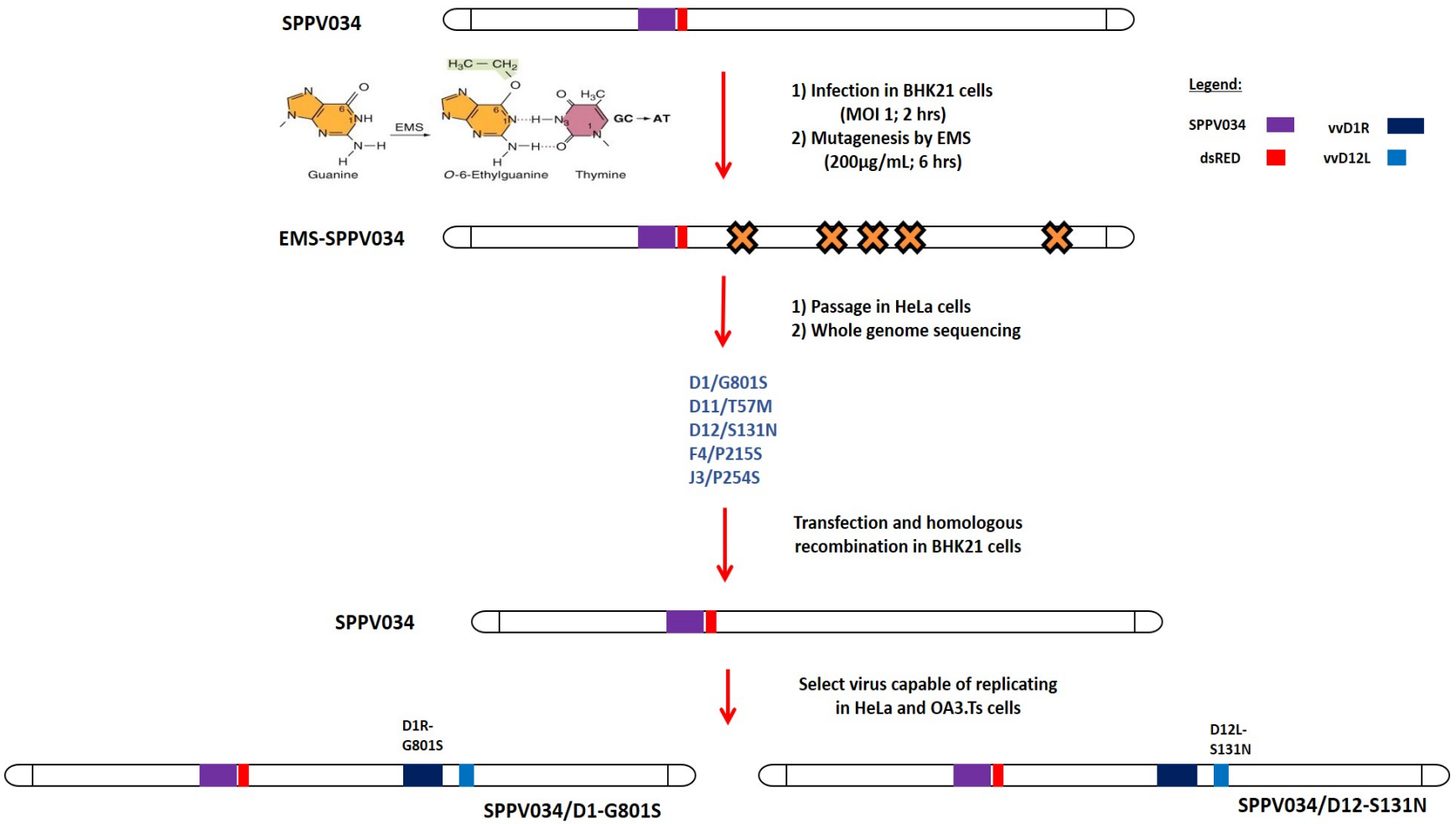

Figure A 2. Schematic of the mutagenesis screen and generation of mutant viruses used.
The SPPV034 recombinant virus was mutated using ethyl methanesulfonate (EMS) and virus was passaged in HeLa cells. Virus capable of replicating were selected and sent for whole genome sequencing. Five potential gene candidates were identified as cofactors for SPPV034. Constructs bearing one mutation in a single candidate gene were created, individually transfected into BHK21 cells and passaged in HeLa cells. Only two mutations were able to individually rescue replication in HeLa cells, the D1-G801S and the D12-S131N mutations. These genes correspond to the large and small subunits of the capping enzyme of vaccinia virus. Mutations were confirmed using a second round whole-genome sequencing.

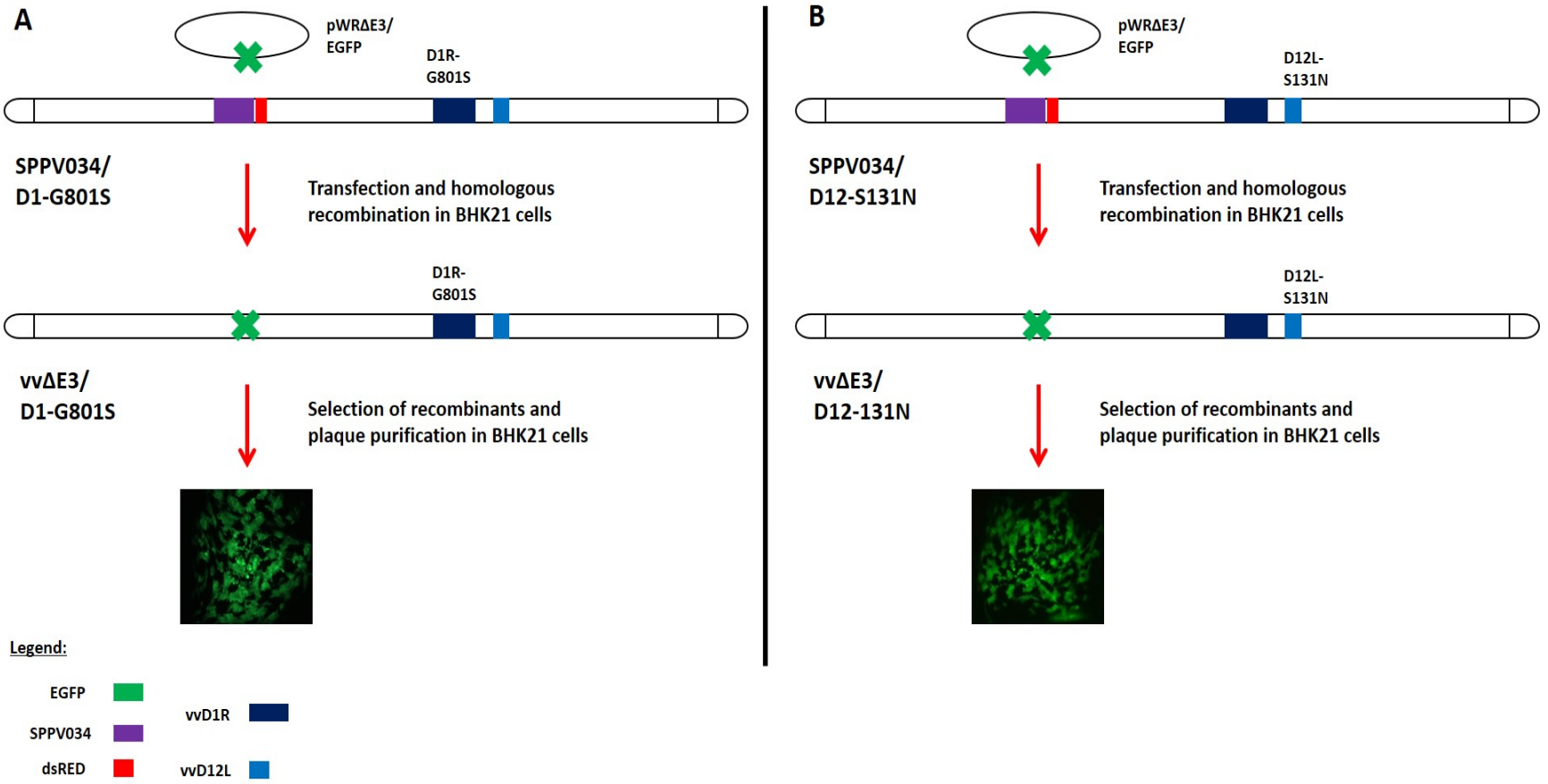

Figure A 3. Schematic of the generation of the knockout viruses bearing mutations in the capping enzyme.
An EGFP fluorescent protein was cloned in between right and left flanking regions, which corresponds to 600 bps of identical sequence to regions flanking the E3L locus of vaccinia virus. The resulting pWRΔE3/EGFP vector was transfected into BHK21 cells infected with either SPPV034/D1-G801S (A) or SPPV034/D12-S131N virus (B). Double crossover events were selected via green only virus plaques and recombinant viruses were purified using three rounds of plaque purification.

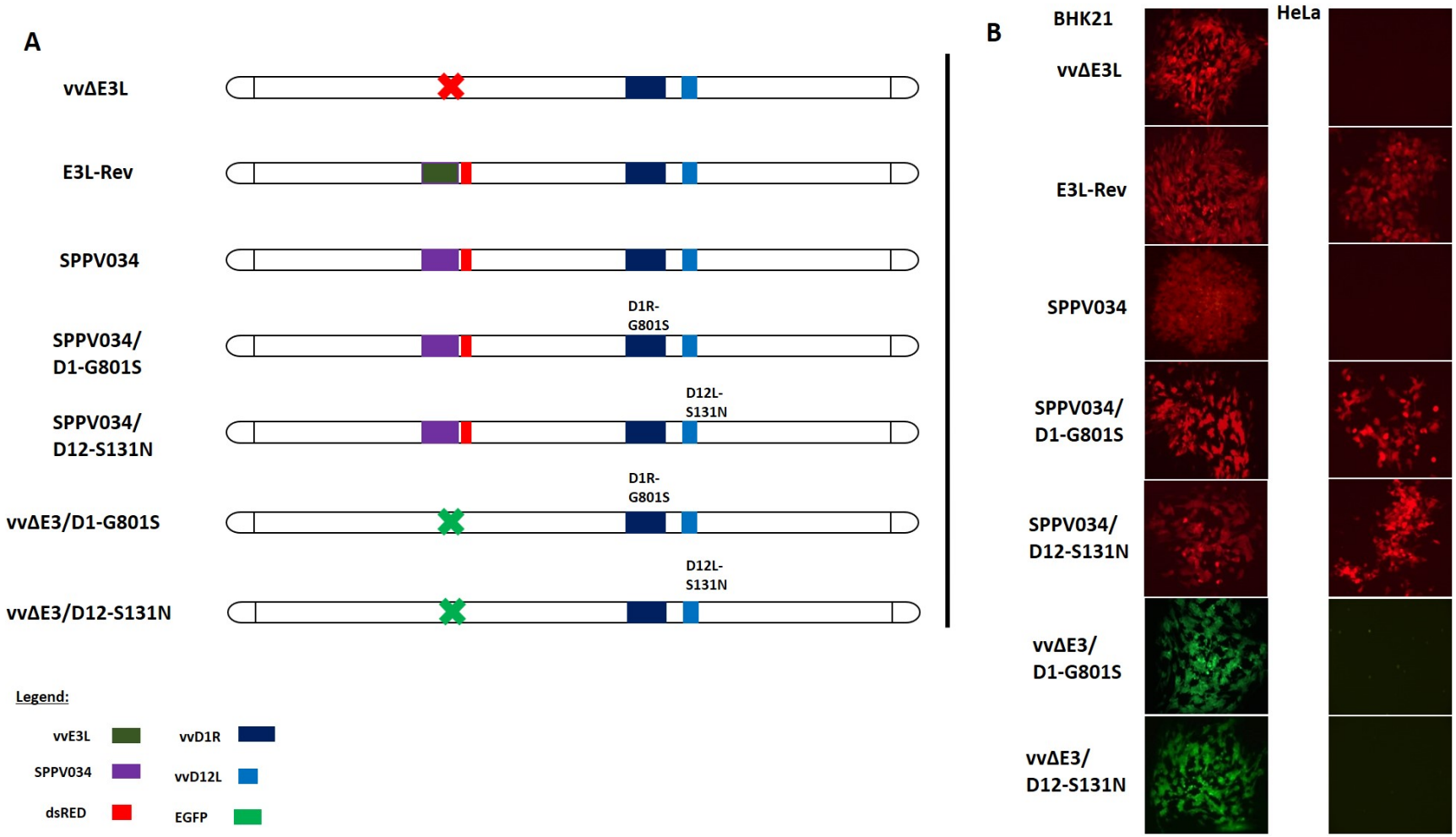

Figure A 4. Depictions of viral constructs used.
A) Genome schematics of recombinant viruses generated for this study. B) Plaque pictures of virus growth in HeLa and BHK21 cells. Cells were infected at MOI 1 and plaques were imaged using a UV microscope at 24 hours post infection.